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Optimization of ZEBRA protein as an innovative delivery system for therapeutic molecules

Roberta Marchione

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THÈSE

Pour obtenir le grade de

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Spécialité : Biotechnologie, Instrumentation, Signal

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dans l'École Doctorale Ingénierie pour la Santé, la Cognition et l'Environnement

Vectorisation de molécules biologiques par la protéine ZEBRA du virus Epstein-Barr: applications en thérapie humaine

Optimization of ZEBRA protein as an innovative delivery system for therapeutic molecules

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*To Andrea,
endless source of enthusiasm and support.*

*Out of clutter, find simplicity.
From discord make harmony.
In the middle of difficulty lies opportunity.
Albert Einstein*

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ABBREVIATIONS

ACPPs	activatable cell-penetrating peptides	NF	eIF3f C-terminal truncation (amino acids 92-361)
AMPs	antimicrobial peptides	OD	optical density
AN	eIF3f N-terminal truncation (amino acids 1-91)	PC	phosphatidylcholine
BCA	bicinchoninic acid	PCI	26S proteasome, COP9 signalosome and eukaryotic initiation factor eIF3 conserved domain
CFU	colony-forming units	PEG	polyethylene glycol
CHO	chinese hamster ovary cell line	PGs	proteoglycans
Chol	cholesterol	PMOs	phosphorodiamidate morphorodiamidate morpholino oligomers
CME	clathrin-mediated endocytosis	PNAs	peptide nucleic acids
CPPs	cell-penetrating peptides	PTDs	protein transduction domains
CS	chondroitin sulfate	RISC	RNA-induced silencing complex
CSLM	confocal scanning laser microscopy	siRNAs	small-interfering RNAs
DBD	DNA-binding domain	SM	sphingomyelin
DIM	dimerization region	TAD	transactivation domain
DMEM	Dulbecco's modified Eagle's medium	TAT	transactivator of transcription
DPBS	Dulbecco's phosphate-buffered saline	Tatp	TAT cell-penetrating peptide
DS	dermatan sulfate	TOP	terminal oligopyrimidine
DTT	dithiothreitol	TP-10	transportan-10
EBV	Epstein-Barr virus	YPD	yeast extract peptone dextrose
eGFP	enhanced green fluorescent protein	ZEBRA	BamHI fragment Z Epstein-Barr replication activator
eIF3	eukaryotic initiation factor 3		
eIF3f	f subunit eukaryotic initiation factor 3		
FACS	fluorescence-activated cell sorting		
FDA	food and drug administration		
GAG	glycosaminoglycan		
HA	hemagglutinin protein		
HIV	human immunodeficiency virus		
HS	heparan sulfate		
IPTG	isopropyl β -D-1-thiogalactopyranoside		
K510R	eIF3f ubiquitination-resistant mutant		
kDa	kiloDalton		
MAP	model amphipathic peptide		
MD	minimal domain		
MPN	Mpr1p and Pad1p N-terminal conserved domain		
mTOR	kinase mammalian target of rapamycin		
mTORC1	mTOR Complex 1		
MTSs	membrane translocation sequences		
M β CD	methyl- β -cyclo-dextrin		

SUMMARY

In recent years, the understanding of disease molecular mechanisms has led to the identification of genes and proteins that are altered in disease state and many therapeutic targets have been found located within cells. The protective and hydrophobic nature of plasma membrane prevents therapeutic drugs from entering cells. Cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) have emerged as a group of non-invasive delivery vectors for various hydrophilic macromolecules, and several *in vitro* and *in vivo* applications as pharmaceutical carriers have been reported. A novel cell-penetrating peptide deriving from the Epstein-Barr virus ZEBRA transcription factor has been recently characterized in our laboratory. A reductionist study of full-length ZEBRA protein has allowed to identify the amino acid region (named as Minimal Domain, MD) implicated in cellular uptake. This peptide is able to cross the mammalian cell membranes via a direct translocation mechanism even when fused to cargo molecules such as eGFP reporter protein. The direct penetration mechanism represents a great advantage for therapeutic applications as the cargo molecules can be directly delivered into cell cytoplasm in a biological active form.

The aim of this thesis is to explore the cell-penetrating properties of the MD peptide and evaluate its applications as therapeutic protein delivery system. This work is structured in three parts.

The first part describes the study on the optimization of MD peptide sequence by size-reduction and the evaluation of its amino acid composition role in the translocation process across the cell membrane. This study has led to the identification of a shorter MD sequence (MD₁₁) with unvaried mechanism of translocation.

The second section describes a MD₁₁-based therapeutic approach aiming at repairing a dysfunction of the protein synthesis identified in most cancers. The regulation of the protein synthesis has a crucial role in governing the eukaryotic cell growth and subtle defects in the translational machinery can alter the cellular physiology and lead to cell malignancy. Among the different factors intervening in the regulation of this process, the eukaryotic initiation factor 3 (eIF3) contributes to oncogenesis and maintenance of the cancer state. This complex is composed of 13 subunits (designated eIF3 a-m). The expression of eIF3 subunits is altered in several cancers, and in particular the f subunit (eIF3f) is significantly downregulated in pancreas, vulva, breast, melanoma, ovary and small intestine tumors. The eIF3f ectopic expression by transient gene transfection inhibits cellular protein synthesis and induces

apoptosis in melanoma and pancreatic cancer cells. Starting from these observations, we have developed an innovative therapeutic approach for cancer treatment in which the missing eIF3f protein is produced *in vitro* in fusion to MD₁₁, and delivered to cells. These results have demonstrated that the MD₁₁- based eIF3f transfer system may represent a powerful strategy to suppress the tumor-cell proliferation.

The last part of this thesis explores the cell-penetrating property of MD₁₁ in yeast cells, and in particular in the pathogenic fungus *Candida albicans*. The presented results demonstrate the versatility of MD₁₁, functioning as vectors in both yeast and mammalian cells and as carrier of biologically active proteins.

The MD₁₁ potential as protein delivery system is evident; however some improvements regarding the fusion protein formulation and *in vivo* studies should be realized to validate the effectiveness of its therapeutic application.

Keywords: Cell-penetrating peptides, protein transduction domains, delivery system, ZEBRA trans-activator, protein therapy, cancer therapy

RESUME

La compréhension des mécanismes moléculaires de différentes pathologies a permis la caractérisation de gènes et de protéines impliqués dans la pathogénèse et l'identification de cibles thérapeutiques intracellulaires. La nature hydrophobique de la membrane cellulaire empêche le passage des médicaments dans les cellules. Les *Cell-Penetrating Peptides* (CPPs) ou domaines de transduction protéiques (PTDs) sont des peptides qui permettent l'internalisation de macromolécules hydrophiles *in cellulo* et *in vivo*. Un nouveau peptide issu du facteur de transcription ZEBRA du virus Epstein-Barr, qui possède des propriétés de transduction, a été caractérisé récemment dans notre laboratoire. Des études par mutagenèse de délétion de la protéine ZEBRA ont permis d'identifier la région d'acides aminés (nommée ainsi MD) impliquée dans la pénétration cellulaire. Ce peptide traverse les membranes des cellules de mammifères par un mécanisme de translocation directe, même lorsqu'il est fusionné à des molécules telles que la protéine reportrice eGFP. Le mécanisme de pénétration directe représente un grand avantage pour les applications thérapeutiques: les molécules cargos peuvent être internalisées directement dans le cytoplasme cellulaire sans dégradation et sous une forme biologiquement active.

L'objectif de cette thèse est d'étudier les propriétés de pénétration cellulaire du peptide MD et d'évaluer ses applications thérapeutiques comme système de vectorisation des protéines. Ce travail est structuré en trois parties.

La première partie porte sur l'étude de l'optimisation de la séquence peptidique MD par réduction de taille et l'évaluation du rôle de sa composition en acides aminés dans le processus de translocation à travers la membrane cellulaire. Cette étude a conduit à l'identification d'une séquence plus courte MD (MD₁₁) possédant une efficacité et un mécanisme de translocation inchangés.

La deuxième partie décrit une approche thérapeutique basée sur MD₁₁ visant à la complémentation protéique d'un dysfonctionnement identifiée dans la plupart des cancers. Les cellules tumorales présentent des altérations dans la machinerie de traduction résultant dans une prolifération cellulaire incontrôlée. Parmi les différents facteurs intervenant dans la régulation de ce processus, le facteur eucaryote d'initiation 3 (eIF3) contribue à l'oncogenèse et au maintien de l'état cancéreux. Ce complexe est composé de 13 sous-unités, désignées eIF3 a-m. L'expression de certaines sous-unités est altérée dans plusieurs cancers, et en particulier la sous-unité f (eIF3f) est significativement diminuée dans le mélanome, les

cancers du pancréas, de la vulve, du sein, de l'intestin et de l'ovaire. L'expression ectopique par transfection transitoire du gène *eIF3f* inhibe la synthèse protéique et induit l'apoptose dans le mélanome et dans les cellules cancéreuses pancréatiques. A partir de ces observations, nous avons développé une approche thérapeutique innovante pour le traitement des cancers dans lesquels la protéine manquante eIF3f est produite sous forme recombinante fusionnée à la séquence de MD₁₁, et ensuite internalisée dans les cellules cibles tumorales. Ces résultats démontrent que le système de transfert de eIF3f basé sur MD₁₁ représente une stratégie efficace pour supprimer la prolifération des cellules tumorales.

La dernière partie de cette thèse explore la propriété de pénétration de MD₁₁ dans les cellules de levure, et en particulier dans le champignon pathogène *Candida albicans*. Les résultats obtenus démontrent la polyvalence de MD₁₁, qui fonctionne comme vecteur de protéines à activité biologique aussi bien dans la levure que dans les cellules de mammifères.

Le potentiel de MD₁₁ comme système de transport et de relargage des protéines a donc été établi, toutefois certaines améliorations en ce qui concerne la formulation des protéines de fusion et des études *in vivo* doivent être réalisées afin de valider son efficacité thérapeutique.

Mots clés: vecteurs de transduction, trans-activateur ZEBRA, domaine de transduction, thérapie protéique, virus Epstein-Barr, thérapie anti-cancéreuse

INTRODUCTION

1. Recent trends in drug development: from small molecules to therapeutics

For many years, the pharmaceutical industry traditionally developed chemical drugs (also referred to as *small molecules*) to treat a wide range of illnesses. Since the 1970s, the drug discovery process has radically evolved, resulting in new therapeutic revolutionary agents: the biomedicines. The main drivers for this transformation are ascribed to progresses made in the field of biotechnology, molecular biology and genetic engineering. The introduction of recombinant DNA and hybridoma technology has provided tools for manipulating genes and cells to produce structurally complex drugs that would have been impossible to manufacture through chemical synthesis or to purify from natural sources (Johnson, 2003). This ability to produce, purify, and characterize complex molecules has correlated with gains in the understanding of disease mechanisms at molecular and cellular level. Indeed, the sequencing and the characterization of the human genome has allowed the identification of various disease-relevant genes and the corresponding disease-linked proteins (Strachan *et al.*, 1999). The combination of these advances has led to the exploration of biological processes involved in the normal and pathogenic states and the discovery of novel intracellular targets, resulting in the development of rationally designed molecular therapies (Chin *et al.*, 2011).

The biotechnology derived drugs range from gene or oligonucleotides to recombinant proteins, and are also named as biomedicines, biologics, biopharmaceuticals, biotherapeutics or biosimilars according to the different jurisdictions (Knezevic *et al.*, 2011). Their high selectivity, potent activity with less side effects, and more predictable behavior, which overcome the conventional drugs (Leader *et al.*, 2008), are leading them to revolutionize the treatment of some of the most difficult-to-treat diseases, prolonging and improving the quality of life and patient care (Knezevic *et al.*, 2011).

2. Therapeutic approaches: gene therapy versus protein therapy

According to the nature of the biologic agent, the variety of resulting therapeutic approaches can be categorized in two main groups: gene therapy and protein therapy.

The term gene therapy describes any procedure intended to treat a disease by genetically modifying the cells of a patient. Because the molecular basis of diseases can vary widely, some gene therapy strategies are particularly suited to certain types of disorders like infectious diseases, cancers, inherited disorders and immune system diseases including allergies and inflammations (Strachan *et al.*, 1999). The rationale of the classical gene therapy is to deliver a therapeutic gene and its associated regulatory elements into the cell nucleus of appropriate target cells with the aim of obtaining optimal expression of the introduced genes (Glover *et al.*, 2005). Depending on the type of disease, the expression of exogenous genes may induce different effects, such as expression of a deficient gene product at physiological levels; inhibition or correction of target gene expression; killing of target cells (*e. g.* cancer cells) by encoding for specific toxins or activating an immune response (Strachan *et al.*, 1999). The most successful gene therapy strategies rely on viral vectors (Hacein-Bey-Abina *et al.*, 2002; Roesler *et al.*, 2002), which can insert unpredictably their DNA into the host genome activating oncogenes or disrupting the expression of tumor-suppressor genes (Schroder *et al.*, 2002; Woods *et al.*, 2003). Due to the difficulties in developing safe and efficient gene-delivery methods, to date gene therapy has been of limited medical benefit (Wilson, 2009).

In protein therapy, the missing or defective protein is produced *in vitro* by recombinant methods and delivered directly into human cells. Indeed, this strategy is considered one of the most direct and safe approach for treating diseases (Yan *et al.*, 2010). Proteins have the most dynamic and diverse role of any macromolecule in the body, catalyzing biochemical reactions, forming receptors and channels in membranes, providing intracellular and extracellular scaffolding support and transporting molecules within a cell or from one organ to another (Leader *et al.*, 2008). Several recombinant human proteins have been developed as therapeutics to replace the natural proteins deficient in some patients (*e. g.* growth hormone), augment existing pathways (*e. g.* interferon- α , and erythropoietin), block specific signaling pathways or deliver radionuclides or cytotoxic drugs (Leader *et al.*, 2008). Human insulin has been the first protein therapeutic deriving from the recombinant DNA technology (Goeddel *et al.*, 1979), and has been approved by the US FDA in 1982. To date, about 100 therapeutic proteins are approved for clinical use in the European Union and the USA, and many more are in preclinical and clinical development (Dimitrov, 2012).

Many of the listed recombinant proteins exert their therapeutic effects through the binding to receptors localized at the cell membrane of organ or tissue of interest. However, when the molecular targets are located intracellularly, therapeutic macromolecules need to gain access into cells to perform their action.

Manufacturing process of recombinant proteins

The complexity of the manufacturing process for biopharmaceuticals is significantly higher than that for small-molecule pharmaceuticals (Schellekens, 2009). Unlike the chemical processes used to synthesize small molecules, therapeutic proteins are manufactured in living cells such as bacteria, yeast, insect cells and plants. This implicates the use of recombinant genetic engineering techniques for cloning of the appropriate genetic sequence into an expression vector, followed by the generation of a host cell expression system and its accommodation for large-scale protein production. The desired protein must then be isolated and purified from the host cells, using purification techniques that maintain the protein's structural and functional integrity (Mellstedt et al., 2008). The purified product must then be correctly formulated, to ensure that it retains its biological activity up to patient delivery.

3. Overcoming barriers to delivery of protein- and nucleic acid- based therapeutics

Regardless the nature of the therapeutic molecules, gene and some protein therapies share a common concept: the delivery of the biologic drug towards its intracellular target. Indeed, to exert their therapeutic action, biological drugs should reach the cytoplasm or individual organelles, such as nuclei (target for gene and antisense therapy), lysosomes (target for the delivery of deficient lysosomal enzymes), and mitochondria (target for pro-apoptotic anticancer drugs) (Torchilin, 2008b).

The plasma membrane is a dynamic structure that segregates the cytoplasm from the extracellular environment. This lipid bilayer is selectively permeable and regulates finely the entry and exit of nutrients and other substances. Its hydrophobic nature impedes the direct passage of ions and polar molecules. Thus, small and nonpolar molecules can easily cross the membrane whereas polar molecules such as amino acids, sugars and ions, can traverse it through the action of integral membrane protein pumps or channels (Conner *et al.*, 2003).

Large macromolecules are carried into the cell in membrane-bound vesicles derived by the invagination of the plasma membrane in a process termed endocytosis (Conner *et al.*, 2003). After the entrapment into endosome vesicles, macromolecules end in lysosomes, where degradation processes take place under the action of numerous digestive enzymes (Conner *et al.*, 2003).

As only compounds within a narrow range of molecular size and polarity passively penetrate into cells, the plasma membrane represents a major limit for the intracellular delivery of hydrophilic therapeutic agents such as nucleic acids or proteins. In addition, even if an efficient cellular uptake via endocytosis is observed, the biological function and the pharmaceutical potency of these macromolecules are compromised by endosomal entrapment and subsequent lysosomal degradation (Torchilin, 2008b).

In recent years, substantial progress has been made to find and design novel technologies for improving the cellular entry of therapeutic compounds, and there is a continuous demand for the development of a variety of tools to overcome intracellular delivery barrier (Heitz *et al.*, 2009). As biotherapeutics have different physical and chemical characteristics (molecular size, stability, conformation, etc.), the specific approach utilized is selected on the basis of the pathological application and class of drug being delivered. Notwithstanding, an ideal drug delivery system should deliver the cargo molecule in specific and challenging cell lines; escape from endosomes; reach the intracellular target; lack of toxicity; and be easy to use for therapeutic applications (Heitz *et al.*, 2009).

Numerous physical methods have been explored to induce intracellular drug delivery, such as ultrasounds (Liu *et al.*, 2012; Prausnitz *et al.*, 2008), electroporation (Prausnitz *et al.*, 2008), magnetic nanovectors (Klostergaard *et al.*, 2012) and photo modulations (Rodriguez-Devora *et al.*, 2012). However, these methods have achieved limited success due to low uptake efficiency (Liu *et al.*, 2012) and high cytotoxic effects (Klostergaard *et al.*, 2012; Prausnitz *et al.*, 2008). Chemical carriers, such as nanospheres, nanocapsules, liposomes, micelles, cell ghosts, lipoproteins, and polymers have been widely used to deliver several therapeutic agents (Koren *et al.*, 2012b).

Liposomes

Among chemical carriers, the most studied drug delivery systems are liposomes. They consist of artificial synthetic phospholipid vesicles that can incorporate membrane proteins (Liguori et al., 2009; Liguori et al., 2008) or can be loaded with a variety of drugs in the interior (Torchilin, 2005). These nanocarriers provide protection to their payload against enzymatic degradation and improve drug solubility (Koren et al., 2012b; Perche et al., 2013; Torchilin, 2008a). However, they lack active targeting and are rapidly cleared upon administration. In addition, their efficacy is often limited by insufficient endosomal escape. Improved efficacy and specificity have been obtained modifying the external surface with moieties such as cell-targeting molecules, hydrophilic polymers, and pH-responsive polymers (Li et al., 2011). Several liposomal drugs have been approved for clinical use, and many are in the various stages of clinical development (Perche et al., 2013; Swami et al., 2012).

Alternative delivery methods consist in the use of peptides, referred to as Cell-Penetrating Peptides (CPPs) or Protein Transduction Domains (PTDs) that are able to translocate the cell membrane. These peptides provide an efficient tool for achieving access to cytoplasm and to subcellular compartments. For the purpose of this thesis, we will concentrate mainly on CPPs as drug delivery system.

4. Cell-penetrating peptides

4.1. Discovery

The proof-of-concept of protein transduction into cells was first described in 1988 in parallel by Frankel and Pabo (Frankel et al., 1988) and Green and Loewenstein (Green et al., 1988) who discovered that the transactivator of transcription (TAT) protein of HIV could cross cell membranes and be efficiently internalized by cells *in vitro*, promoting the viral gene expression. Few years later the transcription factor of *Drosophila melanogaster*, Antennapedia homeodomain, was also shown to enter nerve cells and regulate neural morphogenesis (Joliot et al., 1991). The interesting spontaneous entry of both proteins led to extensive structure/function studies to find the shortest amino acid minimal sequence that enabled cell entry. This resulted in the identification of the first CPPs: Tat peptide, corresponding to the basic domain of HIV-1 Tat protein (Green et al., 1989; Vives et al., 1997) and penetratin (pAntp), corresponding to the third helix of the Antennapedia homeodomain (Derossi et al., 1994). Since then, various peptides showing penetrating capacities have been discovered or

rationally designed (Table 1), such as MTS (Lin et al., 1995), VP22 (Elliott et al., 1997), ZEBRA-MD (Rothe et al., 2010), transportan (Pooga et al., 1998), model amphipathic peptide (MAP) (Oehlke et al., 1998a), signal sequence-based peptides (Lindgren *et al.*, 2000), and synthetic arginine-enriched sequences (Futaki *et al.*, 2001).

Generally, cell-penetrating peptides are defined as a relatively short peptides, 5-40 amino acids, with the ability to gain access to the cell interior by means of different mechanisms, including endocytosis, and with the capacity to promote the intracellular delivery of covalently or noncovalently conjugated bioactive cargoes (Langel, 2006). Their discovery has completely revolutionized the notion of plasma membrane as an impermeable barrier.

4.2. Classification

With a great sequence variety and large differences in terms of physical chemical properties, CPPs can be linear, cyclical, cationic, anionic, hydrophobic, amphipathic, random coiled, α -helical or β -sheets, and use various mechanisms to enter cells (Milletti, 2012). This heterogeneity in sequences, structural properties, and uptake mechanisms makes difficult to elaborate a general definition covering the characteristics of the different CPPs. Thus, several classifications based on different criteria have been proposed.

According to their origin (Lindgren *et al.*, 2000; Zorko *et al.*, 2005), CPPs can be arranged into three classes: protein derived CPPs, model peptides, designed CPPs (Table 1). Protein derived CPPs usually consist of the minimal effective sequence of the parent translocation protein, and are known also as protein transduction domains or membrane translocation sequences (MTSs). Examples are listed in Table 1. Model CPPs comprise sequences that have been designed with the aim of producing well defined amphipathic α -helical structures or of mimicking the structures of known CPPs. Examples are the MAP (Oehlke *et al.*, 1998a) or polyarginine sequences (Futaki *et al.*, 2001). Designed CPPs are usually chimeric peptides composed of hydrophilic and hydrophobic domains of different origin, such as transportan, a fusion peptide of galanin and mastoparan (Pooga *et al.*, 1998).

Table 1. Classes and sequences of some known CPPs. Basic residues (Arg and Lys) are indicated in red.

Class	Origin	Sequence
Protein/peptide derived		
Tat peptide	HIV-1 TAT, residues 48-57	GRKKRRQRRR
Penetratin	Antennapedia homeodomain; residues 43-58	RQIKIWFQNRRMKWKK
VP22	Herpes Simplex virus 1 protein 22; residues 267-301	VDASTATRGRSAASRPTERPRAPARSASRP RRPVE
MTS	Kaposi fibroblast growth factor; residues 129-144	AAVALLPAVLLALLAP
pVEC	Vascular endothelial cadherin; residues 615-632	LLIILRRRIRKQAHASK
MD	Epstein-Barr virus ZEBRA protein, residues 170-220	ECDSELEIKRYKNRVASRKCRAKFKQLLQ HYREVAALKSSENDRLRLLLKQ
Designed		
<i>Model CPP</i>		
MAP		KLALKLALKALKAALKLA
Polyarginines		RRRRRRR (from R7 to R11)
<i>Designed CPP</i>		
MPG		GALFLGFLGAAGSTMGAWSQPKSKRKV
Transportan		GWTLNSAGYLLGKINLKALAALAKISIL

Other attempts to classify CPPs, in spite of their diversity, are based on the physical-chemical properties (Milletti, 2012). Three major CPP classes have been identified: cationic, amphipathic and hydrophobic. Cationic CPPs contain a stretch of positive charges that are essential for uptake without any 3D amphipathic arrangement or hydrophobic character (*e. g.* Tatp and octaarginine). Amphipathic CPPs consist of a polar domain and a hydrophobic domain. The amphipathic character arises from either the primary structure or the secondary structure. Primary amphipathic peptides are defined as the sequential assembly of a domain of hydrophobic residues with a domain of hydrophilic residues (*e. g.* MPG, Pep-1 and pVEC). Secondary amphipathic peptides are generated by the conformational state, which allows positioning of hydrophilic and hydrophobic amino acids in separate faces of the helix (*e. g.* transportan, MAP, GALA, CADY) (Deshayes *et al.*, 2005; Milletti, 2012). Hydrophobic CPPs have a low net charge and possess a hydrophobic motif that is crucial for uptake regardless of the rest of the sequence (*e. g.* Pep-7, BIP) (Milletti, 2012).

Another classification takes into account the relationship between the different peptide sequences and their binding properties to lipids (Madani *et al.*, 2011a). CPPs are grouped in: primary amphipathic, secondary amphipathic and nonamphipathic. Primary amphipathic, such as transportan, interact with both natural and anionic lipid membranes and enter cells via direct membrane transduction. Secondary amphipathic CPPs, such as penetratin and pVEC, typically bind to model membranes with a certain fraction of anionic lipids. Their amphipathic property is revealed when they form an alpha-helix or a beta sheet structure upon interaction with a phospholipid membrane. The nonamphipathic peptides, such as R9 and Tat, bind to the lipid membrane with a high amount of anionic lipids (Madani *et al.*, 2011a).

4.3. Mechanisms of internalization

A full understanding of the physico-chemical interactions and biological processes that determine the uptake, is crucial for further development of CPPs as adaptable and efficient delivery vectors and for their *in vivo* therapeutic applications. To this end, many studies have been conducted in recent years to elucidate the mechanisms by which CPPs enter living cells and mediate the entry of large cargo molecules.

The first reported studies for penetratin, Tat peptide and R₉ indicated that these peptides enter cells by passive, temperature- and receptor- independent process (Derossi *et al.*, 1996; Derossi *et al.*, 1994; Futaki *et al.*, 2001; Vives *et al.*, 1997). It was thus suggested that CPPs penetrate into cells via direct passage through plasma membrane. As the translocation was observed under conditions of low temperature and ATP depletion, endocytic mechanisms were almost ruled out. However, in 2003 a re-evaluation of uptake mechanism was proposed after the discovery that the experimental techniques, routinely employed to study the internalization, led to artifactual redistribution of CPPs into cells (Lundberg *et al.*, 2002; Richard *et al.*, 2003). Thus, the analysis of the cellular mechanisms moved towards observations using living cells (Thoren *et al.*, 2003) and, ever since, the mechanism of many CPPs has been re-examined and reported to be mainly mediated by endocytosis (Fischer *et al.*, 2004; Kaplan *et al.*, 2005; Nakase *et al.*, 2004; Richard *et al.*, 2005; Richard *et al.*, 2003; Wadia *et al.*, 2004). However, evidences for routes of entry independent of the endosomal pathway and involving the trans-membrane potential have also been reported (Deshayes *et al.*, 2005; Henriques *et al.*, 2005; Rothbard *et al.*, 2004; Terrone *et al.*, 2003; Thoren *et al.*, 2003).

Other recent investigations support the notion that cellular delivery can take place by direct plasma membrane translocation in presence of threshold peptide concentration or of hydrophobic cargoes (Duchardt *et al.*, 2007; Fretz *et al.*, 2007; Hirose *et al.*, 2012; Kosuge *et al.*, 2008). Despite the great amount of studies and reports, it remains difficult to establish a general scheme for CPP uptake mechanism. It is now generally accepted that CPP-cargo conjugates first interact with membrane-associated proteoglycans through electrostatic interactions, and are then internalized. Whether endocytosis or direct penetration is involved is still controversial and may depend on several factors (Foged *et al.*, 2008; Heitz *et al.*, 2009; Mueller *et al.*, 2008), including:

- the nature and secondary structure of the CPP;
- its ability to interact with cell surface and membrane lipid components;
- the nature, type and active concentration of the cargo;
- the cell type and membrane composition.

Indeed, positive charged residues in CPP sequence and hydrophobic alpha helical structures influence positively the uptake efficiency (Mitchell *et al.*, 2000; Oehlke *et al.*, 1998b; Scheller *et al.*, 1999). In particular, the guanidinium groups of arginine residues contribute to translocation more efficiently than lysine side chains (Mitchell *et al.*, 2000). The CPP sequence and conformation also play a role in internalization mechanism. For example, pVEC efficiently translocates into various cell lines whereas scrambled pVEC has minor uptake (Mueller *et al.*, 2008). Even the CPP concentration may impact the entry mechanism: direct penetration is more probable for primary hydrophobic CPPs at high concentrations, whereas endocytosis is the main uptake mechanism at low concentrations. Furthermore, the concentration threshold for direct penetration varies between different CPPs, cell lines, the presence and type of cargo (Madani *et al.*, 2011b). Type, size and coupling methodology of a cargo affect the translocation mechanism. For example, Tat peptide coupled to a large cargo is mostly entrapped in the endosomal vesicles; however it redistributes throughout the cell cytoplasm when attached to a small cargo (Tunnemann *et al.*, 2006). Different modes of uptake are also determined by lipid composition, density and dynamics of cell membrane (Thoren *et al.*, 2004). Several evidences also suggest the existence of a multiplicity of entry pathways that may occur simultaneously depending on the CPP (Duchardt *et al.*, 2007; Jiao *et al.*, 2009).

4.3.1. Interaction with cell-surface proteoglycans

In the majority of cases, internalization begins with interactions between the CPPs and the extracellular matrix, requiring the capture of the peptides by cell-surface proteoglycans (PGs). Proteoglycans are a heterogeneous group of proteins with covalently bound linear and negatively charged glycosaminoglycan (GAG) polysaccharides. Chondroitin sulfate (CS), dermatan sulfate (DS) or heparan sulfate (HS) are the most prevalent GAGs in PGs.

The electrostatic interaction between PGs and cationic CPPs (*e. g.* Tat and polyarginine) is considered the first step for their successful translocation. Specifically, the reduction in uptake observed after enzymatic removal of heparan sulfate chains by heparinase, and the poor internalization of CPPs in mutant chinese hamster ovary (CHO) cell lines lacking either all GAGs or only HS (Duchardt *et al.*, 2009; Nakase *et al.*, 2007; Richard *et al.*, 2005; Wallbrecher *et al.*, 2013) have demonstrated that PGs play a major role in cationic peptide uptake. The number of positive charges and, in particular, the number of arginines is critical for the CPP interaction with PGs (Nakase *et al.*, 2007). Along with electrostatic forces, also hydrogen-bonding and/or hydrophobic interactions are involved in the interaction. For example, hydrophobic interactions are critical for stabilizing the binding to sulfated sugars and for facilitating clustering at cell surface of two penetratin variants differing in the numbers of arginines and lysines (Amand *et al.*, 2012). Moreover, for a series of penetratin analogs, it has been demonstrated a direct correlation of the tryptophans number in the interaction with GAGs and in the internalization efficiency (Bechara *et al.*, 2013a). Therefore, with regard to the molecular mechanisms of these interactions, arginine residues contact the sugar units either by electrostatic and bidentate hydrogen interactions with the sulfates, while tryptophan residues bind to sugars rings by hydrophobic interactions (Bechara *et al.*, 2013b).

For amphipathic peptides such as transportan-10 (TP-10), much less is known about the role of HS in cellular uptake. Even in absence of arginine residues, TP10 is able to form clusters with HS chains. This interaction with HS is thermodynamically and functionally different from the one described for arginine-rich CPPs (Verdurmen *et al.*, 2013). By labeling sialic acids in the glycocalyx and by monitoring the colocalization of peptides on the cell surface, it has been demonstrated that HS chains have the capacity to sequester amphipathic CPP in the glycocalyx in a manner that is unproductive for uptake (Verdurmen *et al.*, 2013).

Generally, PGs act as cellular attachment sites for CPPs and facilitate their accumulation in clusters at the cell surface, that triggers the remodeling of the actin network and a selective activation of the GTPase involved in endocytosis (Nakase *et al.*, 2007; Poon *et al.*, 2007; Ziegler *et al.*, 2011). Thus, in presence of specific structural requirements for binding and induction of uptake, GAGs may be considered as CPP receptors (Favretto *et al.*, 2014). The adhesion of CPPs with cell-surface PGs has also been correlated to the physiological cell-membrane recycling (Brooks *et al.*, 2005). Cells nonspecifically internalize and recycle the equivalent of their surface one to five times per hour (Steinman *et al.*, 1983). Therefore, peptides with strong affinity for PGs adhere and accumulate on cell-surface, and can be partially internalized through natural cell-membrane turnover simultaneously to other entry mechanisms.

4.3.2. Translocation through the cell membrane

After binding to PGs, CPPs can reach the cell interior by two mechanisms, broadly categorized in energy-dependent endocytosis and energy-independent direct translocation. As mentioned above, it does not exist a unified pathway for all CPPs due to the presence of several factors that can influence this process. The existing data are at times controversial and may lead to ambivalent interpretation. The results obtained with free CPPs may not correlate precisely with the results with CPP-cargo complexes or when comparing different cargoes with one another. Even when endocytosis is identified as main internalization pathway for a defined CPP, the employed endocytic route might change on the basis of the transported cargo, the concentration, the cell-type, etc. Thus, in the following section a general description of the endocytic pathways will be presented, extrapolating only few examples of well-known CPP imports among the many known.

4.3.3. Endocytosis mechanisms

Endocytosis represents the conventional mode of cellular entry for hydrophilic macromolecules, and involves absorption to the plasma membrane or a membrane-bound receptor, followed by energy-dependent formation of vesicles (Lindgren *et al.*, 2000).

Endocytosis comprises distinct pathways, which can be subdivided into two groups: phagocytosis or “cell eating” and pinocytosis or “cell drinking”. Phagocytosis relates to the

uptake of large particles and is typically restricted to specialized mammalian cells, such as macrophages, monocytes, and neutrophils that are specialized for the elimination of pathogens and apoptotic or infected cells (Conner *et al.*, 2003). Pinocytosis occurs in all cells and encompasses a variety of processes leading to the uptake of fluids, solutes, and membrane components. At least four different pinocytic pathways can be distinguished: macropinocytosis, caveolae-mediated endocytosis, clathrin-mediated endocytosis (CME) and clathrin- and caveolin- independent endocytosis (Conner *et al.*, 2003).

To clarify the involvement of these internalization pathways in CPPs uptake, different experimental approaches have been employed, such as:

- analysis of peptide/cell interaction at low temperatures (approximately 4°C) or in energy depletion conditions;
- incubation with drugs that selectively compromise different internalization pathways;
- evaluation of peptide or peptide conjugate co-localization with molecules known to be internalized by specific endocytotic pathways (*e. g.*, transferrin, cholera toxin G subunit) or with molecular markers of known internalization pathways (*e. g.*, caveolin-1, early endosome antigen-1),
- internalization in cells lacking functional clathrin-mediated or caveolae-dependent internalization pathways.

All known types of endocytosis have been reported to participate in the cellular uptake of several CPP-cargo complexes (Table 2).

Table 2. Summary of mechanisms reported for endocytosis of some known CPPs.

Mechanism	PTD/ CPP	Evidence provided	Cell line
Macropinocytosis	Tat-Cre (Wadia <i>et al.</i> , 2004)	Uptake inhibition at 4°C and in presence of amiloride, cytochalasin D, nystatin, MβCD; lack of inhibition in presence of dominant-negative mutant of dynamin; co-localization with dextran	reporter T-cells
	Oligoarginine peptides (R8) (Nakase <i>et al.</i> , 2004)	Uptake inhibition at 4°C and in presence of cytochalasin D, amiloride; induction of cytoskeleton rearrangement	HeLa
	Tat peptide (Kaplan <i>et al.</i> , 2005)	Uptake inhibition at 4°C and in presence of amiloride, cytochalasin D, MβCD; co-localization with dextran	Namalwa lymphoma cells
	R8-liposomes (Khalil <i>et al.</i> , 2006)	Uptake inhibition in presence of amiloride, cytochalasin D, nystatin; lack of co-localization with transferrin or LacCer; co-localization with dextran	NIH-3T3
	Tat-Streptavidin (Rinne <i>et al.</i> , 2007)	Uptake inhibition in presence of cytochalasin D and amiloride; lack of inhibition by methyl-β-cyclodextrin; co-localization with early endosomal and lysosomal markers; lack of co-localization with caveolin; internalization in HepG2 cells	HeLa
	pVEC-, M918-, penetratin- coupled to luciferin (Mager <i>et al.</i> , 2012)	Uptake inhibition in presence of cytochalasin D	HeLa
Caveolae-dependent endocytosis	GST-Tat-eGFP (Ferrari <i>et al.</i> , 2003; Fittipaldi <i>et al.</i> , 2003)	Uptake inhibition at 4°C and in presence of MβCD and cytochalasin D; co-localization with dextran and caveolin; lack of co-localization with transferrin and Lysotracker	COS-1, HeLa
	Transportan-avidin/neutravidin complexes (Saalik <i>et al.</i> , 2009)	Uptake inhibited after caveolin silencing and at 4°C, co-localization with early endosomal marker, caveolin and transferrin; lack of inhibition after flotillin-1 silencing	HeLa
Clathrin-mediated endocytosis	Tat peptide (Richard <i>et al.</i> , 2005)	Uptake inhibition at 4°C and in presence of chlorpromazine or potassium depleted medium, lack of inhibition by filipin or nystatin; uptake observed in HepG2 cells	CHO, HeLa
	Tat-Streptavidin (Rinne <i>et al.</i> , 2007)	Uptake inhibition in presence of cytochalasin D and amiloride; lack of inhibition by methyl-β-cyclodextrin; co-localization with early endosomal and lysosomal markers; lack of co-localization with caveolin; internalization in HepG2 cells	HeLa

Amiloride: Na⁺/H⁺ exchange inhibitor; Chlorpromazine: clathrin depletion from plasma membrane; Cytochalasin D: F-actin polymerization inhibitor; Dextran: fluid-phase marker; Dynamin: clathrin- and caveolar-mediated endocytosis marker; Filipin: binding to plasma membrane cholesterol; Flotillin: lipid-raft marker; HepG2 cells: human hepatoma cells deficient in caveolin-1; LacCer: caveolae marker; Lysotracker: marker for cell lysosomes; MβCD: cholesterol depletion from plasma membrane; Nystatin: binding to plasma membrane cholesterol; Transferrin: Clathrin-mediated endocytosis marker

Macropinocytosis, which is induced in many cell types upon stimulation by growth factors or other signals, has been reported to play a significant role in the uptake of several CPPs (Table 2). The signaling cascades inducing macropinocytosis involve Rho-family GTPases, which trigger the actin-driven formation of membrane protrusions. These protrusions do not “zipper up” along a ligand-coated particle, but instead they collapse onto and fuse with the plasma membrane (Figure 1) to generate large endocytic vesicles, called macropinosomes, that entrap large volumes of the extracellular milieu (Conner *et al.*, 2003). Using a transducible Tat-Cre recombinase reporter assay on living cells, Wadia *et al.* demonstrated that macropinocytosis is the main mechanism of internalization of this fusion protein (Wadia *et al.*, 2004). Macropinocytosis also play an important role in cellular uptake of fusion protein Tat-Streptavidin (Rinne *et al.*, 2007), oligoarginine peptides (Nakase *et al.*, 2004), liposomes-octaarginine peptides (R8) (Khalil *et al.*, 2006), and pVEC-, M918-, penetratin- coupled to luciferin (Mager *et al.*, 2012).

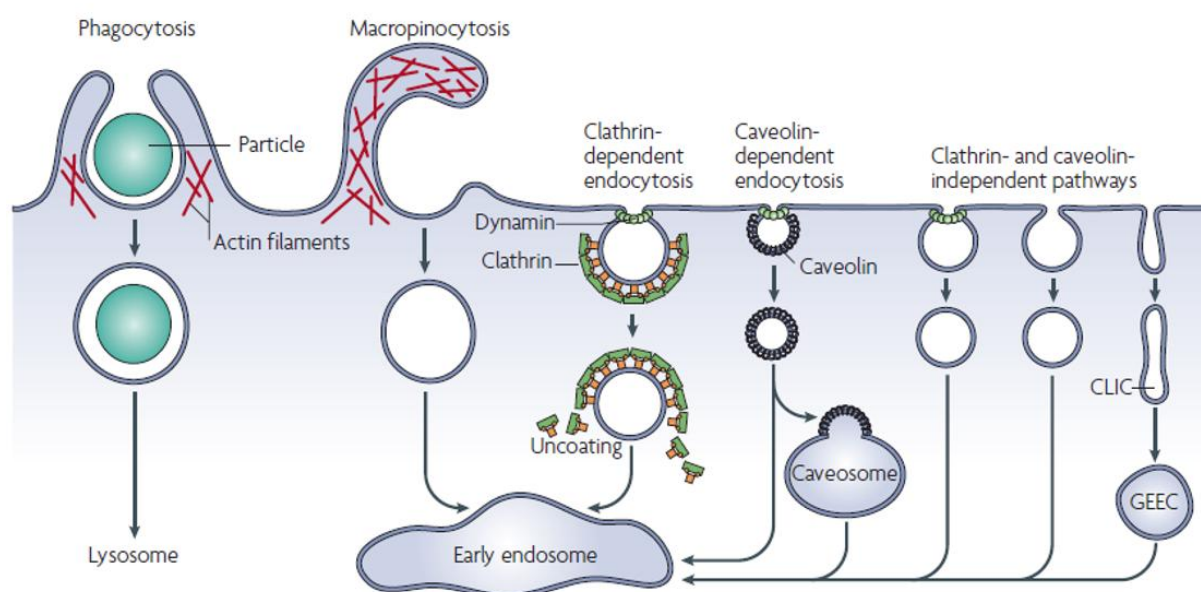


Figure 1. Multiple portals of entry into mammalian cells. The endocytic pathways differ with regard to the size of the endocytic vesicle, the nature of the cargo (ligands, receptors and lipids) and the mechanism of vesicle formation. Once internalized, vesicles containing cargoes molecules are delivered to early endosomes (Mayor *et al.*, 2007).

Unconjugated Tat peptide (Richard *et al.*, 2005) and fusion proteins containing Tat-, R10- and K10- coupled to the tetramerization domain of human p53 (Kawamura *et al.*, 2006), are also internalized by clathrin-mediated endocytosis. CME occurs constitutively in all mammalian cells, and allows the internalization of proteins and other molecules into the cell through the use of specific receptors expressed on the cell surface (Watson *et al.*, 2005). After the binding of an extracellular ligand to specific cell-surface receptors, clathrin together with other adapter proteins builds endocytic “coated pits” at the plasma membrane (Figure 1). Coated pits invaginate and pinch off to form cargo-filled vesicles encapsulated by a polygonal clathrin coat (few hundred nanometers in diameter) (Conner *et al.*, 2003).

The participation of caveolae-dependent endocytosis has been reported in the internalization of fusion proteins GST-Tat-GFP (Ferrari *et al.*, 2003; Fittipaldi *et al.*, 2003), and transportan- avidin/neutravidin complexes (Saalik *et al.*, 2009). Caveolae are flask-shaped invaginations of the plasma membrane present on many mammalian cells, with a smooth surface and a diameter of 55-65 nm. Caveolae are known to demarcate cholesterol and sphingolipid-rich microdomains of the plasma membrane, in which many diverse signaling molecules and membrane transporters are concentrated (Anderson, 1998). The shape and structural organization of caveolae are conferred by caveolin, a dimeric protein that binds cholesterol, inserts as a loop into the inner leaflet of the plasma membrane, and self-associates to form a striated caveolin coat on the surface of the membrane invaginations (Figure 1) (Conner *et al.*, 2003).

Caveolae- and clathrin-independent endocytosis fulfill unique functions in the cell and vary mechanistically not only in the way by which the vesicles are formed, but in terms of which cargo molecules they transport, to what intracellular destination their cargo is delivered, and how their entry is regulated (Conner *et al.*, 2003). The mechanisms that govern caveolae- and clathrin-independent endocytosis remain poorly understood. Caveolae represent just one type of cholesterol-rich microdomain on the plasma membrane. Others, more generally referred to as “rafts”, are small structures that diffuse freely on the cell surface and are implicated in specific sorting of membrane proteins and/or glycolipids (Edidin, 2001). By investigating the co-localization of penetratin and Tat peptides with cholera toxin (a marker of lipid rafts), it has been reported an internalization via lipid raft-dependent but clathrin-independent endocytosis for both peptides (Jones *et al.*, 2005).

4.3.4. Sequestration in the endosomal compartment

After endocytic capture at the plasma membrane, molecules engulfed in endosomal vesicles are subject to rapid sorting into different compartments tagged for destruction or recycling. Depending on the nature and the concentration, the internalized material can be directed to different cellular destinations. For example, the classical clathrin-mediated pathway recycles some of its contents back to the plasma membrane, whereas most of the materials from the clathrin-coated vesicles are targeted to late endosomes and lysosomes for degradation (Conner *et al.*, 2003; Lakadamyali *et al.*, 2006). Macropinosomes are also considered to target their contents to lysosomal degradation (Racoosin *et al.*, 1993). In CPP-mediated protein delivery, the recycling pathway plays only a negligible role whereas the majority of protein complexes follows the endolysosomal pathway after internalization (Raagel *et al.*, 2009). Molecules following the endolysosomal pathway are entrapped in early endosomes and follow a step-by-step maturation process until a final degradation in lysosomes at low pH. Many groups have shown that most of the CPP-cargoes are found in large acidic compartments (Al-Taei *et al.*, 2006; Padari *et al.*, 2005; Sandgren *et al.*, 2002; Vendeville *et al.*, 2004), where the hydrolytic enzymes break down the complexes. The inhibition of endosomal acidification (by adding of lysosomotropic agents like chloroquine) increases the bioactivity of the cargo molecules resulting in the enhanced interaction of the cargoes with their intracellular targets (Abes *et al.*, 2007; El-Andaloussi *et al.*, 2006). The gradual trafficking of the CPP-cargoes to the lysosomal compartments and the successive degradation strongly impair the potential of CPPs as effective transporters and represent a limiting factor for those applications in which efficient transport to cytoplasm or particular cellular organelles is required.

4.3.5. Endosomal escape

The events inside the endosomes that could enhance the escape of the CPP-cargo complexes are still not defined. Vendeville *et al.* have demonstrated that the gradual drop of pH is needed to trigger the conformational changes that lead to membrane insertion and subsequent translocation of TAT protein (Vendeville *et al.*, 2004). Using artificial liposomes prepared from the lipid mixture mimicking the composition of the late endosomes, Abes *et al.* have found that the acidic pH of 5.5 induces a stronger leakage than at neutral pH of the CPP-

PMO conjugates out of the entrapping vesicles (Abes *et al.*, 2008). Several strategies have been conceived for increasing endosomal escape. For example, the N-terminal part of the influenza virus hemagglutinin protein (HA, a peptide that destabilizes lipid membranes at low pH (Han *et al.*, 2001)), has been efficiently used to promote the endosomal escape of Tat-Cre fusion proteins entrapped into macropinosomes. The pH drop facilitates the conformational changes in the HA and contributes to the insertion of the complex into the lipid bilayer of the endosomal vesicle (Wadia *et al.*, 2004). In addition, the inhibition of Tat peptide release from the endosomes has been demonstrated upon an increase of endosomal pH by NH_4Cl (Potocky *et al.*, 2003) or chloroquine (Fischer *et al.*, 2004), providing evidence that endosomal acidification is a prerequisite for effective CPP escape. However, this kind of chemical reagents may not be suitable for *in vivo* therapeutic use, since their effective concentrations are often associated with high cytotoxicity (Jamshidzadeh *et al.*, 2007).

Another technique used for triggering endosomal escape consists in photochemical treatment of cells co-incubated with CPP-cargoes and photosensitizers. Upon photostimulation, the photosensitizer produces reactive oxygen species which cause damage to endosomal membrane and the consequent release of the cargo into cytoplasm. This approach has been successfully used for increasing bioactivity of peptide nucleic acids by Tat, oligoarginine and KLA peptides (Shiraishi *et al.*, 2006). Compared to other endosomal escape agents, this technique has minor cytotoxicity, but its utilization is limited from effective tissue penetration depths of radiation.

4.3.6. Direct translocation

Evoked at first as the mechanism of internalization of CPPs, then refuted as an artifact of fixation, the existence of direct translocation was later confirmed using fluorescence in living cells, quantification of the uptake at 4°C (which inhibits the majority of energy-dependent pathways) and using model systems (Binder *et al.*, 2003; Jiao *et al.*, 2009; Saalik *et al.*, 2011; Thoren *et al.*, 2004). Direct penetration via energy-independent pathways involves stable or transient destabilization of the plasma membrane, in an energy- and temperature-independent manner. The models proposed to explain the direct translocation across biological membranes include the “inverted micelle formation”, “pore formation”, “carpet-like model”, and “adaptive translocation” (Figure 2).

The first step in all these mechanisms consists in the interaction of the positively charged CPPs with negatively charged components of membranes, such as HS or the phospholipid bilayer. The subsequent mechanism of internalization depends highly on the peptide concentration, peptide sequence, and lipid composition. For example, internalization occurs through direct penetration at high CPP concentrations, and for primary amphiphatic CPPs, such as transportan analogues and MPG (Deshayes *et al.*, 2006; Duchardt *et al.*, 2007; Kosuge *et al.*, 2008).

The direct peptide translocation is often associated to spatially confined areas of the plasma membrane through a mechanism involving the transient formation of pores or continuous transfer across the lipid bilayer (Duchardt *et al.*, 2007; Hirose *et al.*, 2012). Accumulation of negatively charged membrane components and ceramide-enriched domains has been reported in proximity of the peptide influx sites (Duchardt *et al.*, 2007; Hirose *et al.*, 2012; Verdurmen *et al.*, 2010).

The “inverted micelle” formation is the first model proposed to explain the direct translocation of penetratin (Derossi *et al.*, 1996). In addition to the initial electrostatic interaction, the CPP hydrophobic residues interact with the hydrophobic membrane core and induce a destabilization of the bilayer forming a negative curvature (Figure 2A) (Alves *et al.*, 2008). The concomitant reorganization of the neighboring lipids leads to the formation of the inverted micelles that encapsulate the peptide in their interior (Joanne *et al.*, 2009). The interaction with the membrane components leads to an inverse process, resulting in the destabilization of the inverted micelles and the consequent release of the peptides into the intracellular compartment (Figure 2A). The driving force for this event is the electrostatic field created by the differential peptide concentrations inside and outside the membrane (Binder *et al.*, 2004).

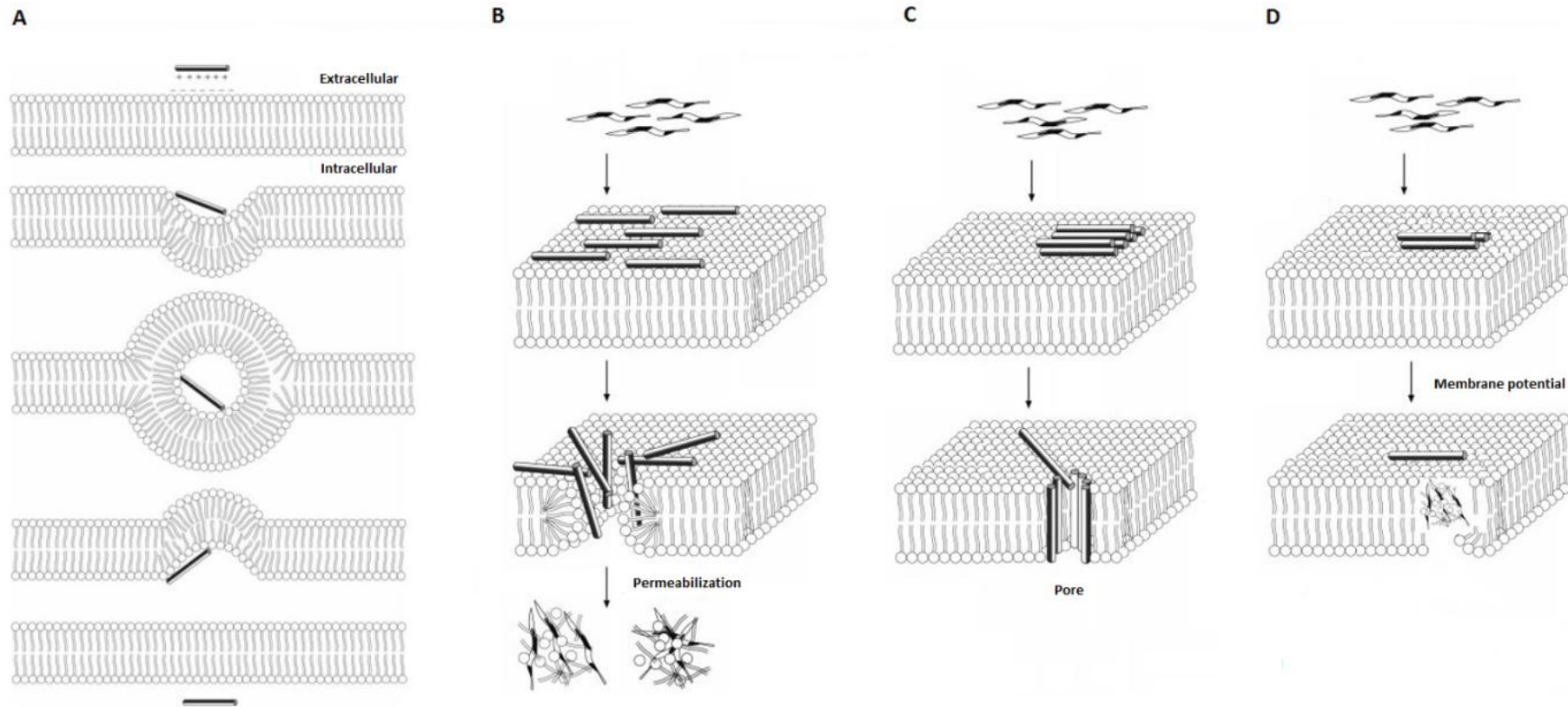


Figure 2. Examples of the proposed mechanisms for direct translocation. A) Inverted micelles formation, B) Carpet-like model, C) Pore-formation, D) Adaptive translocation. Modified image from (Lundberg *et al.*, 2003).

By analogy with the mechanisms of membrane disturbance initially proposed to explain the translocation of antimicrobial peptides and toxins, alternative models have been described for explaining CPP uptake. “Pore formation” involves the formation of “barrel stave” or “toroidal” transient pores that enable passive diffusion of Tat (Herce *et al.*, 2007), MPG (Deshayes *et al.*, 2004a), Pep-1 (Deshayes *et al.*, 2004b) and arginine-rich peptides (Herce *et al.*, 2009) across the plasma membrane (Figure 2C). Pore formation is achieved when CPP concentration is above a threshold concentration, which is different for each peptide. In “barrel stave” pores, the peptides assume an amphipathic alpha-helix structure when inserted into the membrane. The hydrophobic face of the amphipathic helices interacts with the aliphatic chains of the bilayer phospholipids and the hydrophilic face forms the interior of the pore (Lundberg *et al.*, 2003; Shai, 1999). In “toroidal” pores model, the accumulation of peptides in the outer leaflet causes a thinning of the bilayer. The attraction between arginine and lysine side chains and the headgroups of the distal lipid layer leads to the formation of a transient pore through which other peptides diffuse carrying with them the attached phospholipids (Herce *et al.*, 2007; Herce *et al.*, 2009). As consequence of transient membrane disruption, the cytoplasmic concentration of calcium ions increases, and activates a membrane-repair response by exocytosis of intracellular vesicles that fuse with the broken bilayer and reseal it (Palm-Apergi *et al.*, 2009).

According to the “carpet model”, peptides interact with the negatively charged phospholipids, covering the cell surface in a carpet-like manner (Figure 2B). Membrane permeation occurs only in presence of a high local concentration of membrane-bound peptides. Contrary to the “pore formation” model, peptides are not inserted into the hydrophobic core of the membrane, neither they assemble with their hydrophilic surfaces facing each other (Shai, 1999). The membrane translocation of peptide-cargo conjugates occurs as a consequence of a transient destabilization of the cellular membrane, induced by a partial change of peptide secondary structure, and consequent phospholipid reorganization (Lundberg *et al.*, 2003).

According to the “adaptive translocation” model, the positively-charged peptides recruit the negatively-charged membrane constituents at cell surface. This phenomenon induces the transient formation of an ion pair complex with attenuated polarity that is able to adaptively

diffuse into the membrane and subsequently into the cell (Figure 2D) (Wender *et al.*, 2008). The driving force of this mechanism is the membrane potential (Wender *et al.*, 2008).

Although these models share several common features, it is important to highlight some significant differences (Trabulo *et al.*, 2010):

- According to the “inverted micelle model”, the peptides remain associated to the membrane surface during translocation and do not contact the hydrophobic interior of the lipid bilayer, in contrast to what is described in other models where the insertion of the peptides in the membrane and the resulting transmembrane conformation are important steps of the translocation process.
- Both the “toroidal” pore and the “carpet model” describe an extensive reorganization of membrane phospholipids, in contrast to the “barrel stave” model, in which the structure of the lipid bilayer would not be significantly disturbed.
- The models involving the formation of pores, in which peptides oligomerize and insert into membrane, predict the existence of a well-defined structure, contrary to the highly disorganized structure responsible for the destabilization of the cellular membrane described in other models.

With the exception of the “inverted micelle” model, all reported models are compatible with the translocation of large size molecules across biological membranes and require the presence of amphipathic alpha-helix secondary structures (Trabulo *et al.*, 2010).

4.4. Intracellular delivery using CPPs

CPPs have been successfully used to mediate the intracellular delivery of a variety of molecules that do not cross biological membranes by themselves, such as small molecules, oligonucleotides, plasmid DNA, peptides, proteins, nanoparticles, lipid-based formulations, viruses, quantum dots, and contrast agents for magnetic resonance imaging. The number of reports describing CPP applications *in vitro* and *in vivo* is consciously increasing and a great amount of CPP-based strategies have been reported (Heitz *et al.*, 2009). With the aim to highlight the diversity and the versatility of this methodology, some examples of CPP applications, ranging from diagnostic to therapeutic purposes, are reported in this section.

In general, two strategies for coupling cargo molecules to CPPs are used, *i. e.* non covalent complexes formation and covalent coupling (Heitz *et al.*, 2009). The first strategy relies on electrostatic interactions to form complexes in which the positively charged CPPs surround cargo molecules (like oligonucleotides or proteins), masking their negative charges. CPPs are typically used in a molar ratio excess to neutralize negative charges, yielding complexes of various sizes. For covalent coupling, cargo molecules and CPPs are connected via a chemical linker, such as a disulfide bond. After internalization, the bond is broken in cytoplasmic reducing environment and the cargo is released into cells. However, with protein cargoes, the chemistry of linking becomes complicated given the presence of multiple functional groups and the risk of altering the biological activity of the cargoes. Therefore, in most cases the conjugates are prepared by recombination as fusion proteins.

4.4.1. Small molecule delivery

The conjugation of CPPs to small molecule cytotoxic agents has been explored with the goal of modifying their *in vivo* distribution and improving the drug efficacy profile. In such one example, the anticancer drug doxorubicin has been conjugated to penetratin and SynB1 peptides and displayed enhanced uptake across the blood-brain barrier, resulting in increased cytotoxicity towards multi-drug-resistant tumor cells (Rousselle *et al.*, 2000). In a second example, a heptamer of arginine has been conjugated to cyclosporin A (R7-CsA), which in unconjugated form fails to penetrate skin. The R7-CsA is efficiently transported into cells in mouse and human skin and inhibits cutaneous inflammation (Rothbard *et al.*, 2000). This product has entered phase II trials in 2003 for the treatment of psoriasis under the commercial name of PsorBan® (Table 5).

4.4.2. Development of imaging agents

CPPs have been applied for diagnostic purposes in the delivery of probes for *in vivo* fluorescence imaging and tumor visualization during surgery (Nguyen *et al.*, 2010). For instance, a fluorescently labeled CPP has been coupled via a cleavable linker to a neutralizing peptide (Figure 3A). Upon exposure to tumor-tissue specific proteases, the linker is cleaved, dissociating the inhibitory peptide and allowing the CPP to enter tumor cells and defining

then tumor margins (Nguyen *et al.*, 2010). Another recent diagnostic application of CPPs involves the detection of viral infections in living cells (Sivaraman *et al.*, 2013; Yeh *et al.*, 2008). This method is based on the Tat peptide-mediated delivery of molecular beacons (Figure 3B), which are single-stranded, fluorescently labeled oligonucleotide probes that recognize specific viral nucleotide sequences and produce fluorescence upon target binding (Sivaraman *et al.*, 2013; Yeh *et al.*, 2008).

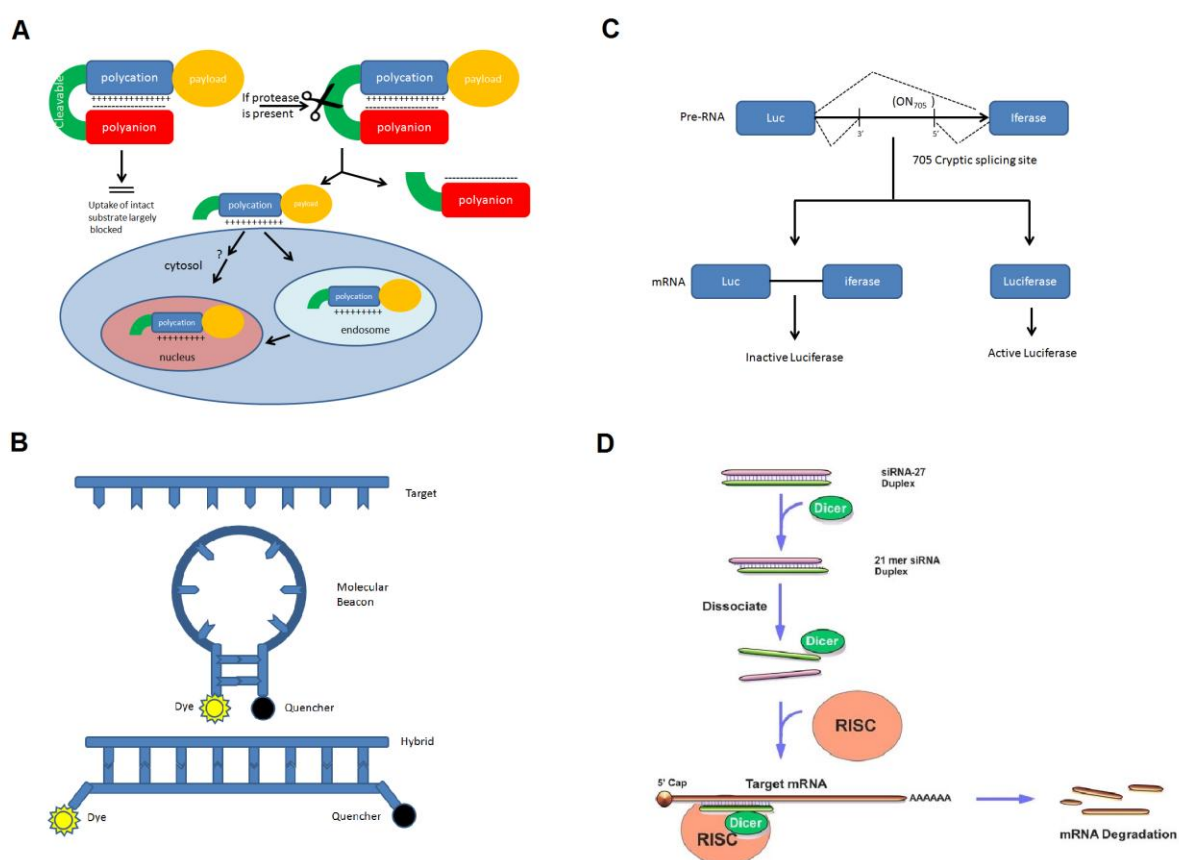


Figure 3. Examples of CPP-based strategies. A) Activatable cell-penetrating peptides (ACPPs). The fluorescently labeled polycationic CPP is coupled via a cleavable linker to a neutralizer. When the peptide is exposed to the proteases characteristic of the tumor cells, the linker is cleaved and the CPP enters tumor cells, providing fluorescence (Modified image from Dr. Roger Tsien). B) Molecular beacons are oligonucleotide probes that emit fluorescence when hybridized to a target sequence of DNA or RNA. On the end of one arm, a fluorescent moiety is quenched by a moiety covalently attached at the end of the other arm. When the probe encounters its target sequence, conformational changes lead to an increase in fluorescence. C) Splicing redirection. Aberrant splicing leads to the production of a non-functional protein. The hybridization of an oligonucleotide to the splice site restores the correct protein expression. Image from (Abes *et al.*, 2011). D) mRNA silencing. Double-strand siRNA is processed by DICER and associates with RISC. Their hybridization to the target mRNA induces specific gene silencing. Copyright © 2014 OriGene Technologies.

4.4.3. Nucleic acid delivery

In the context of gene delivery, CPPs have been explored as safe system for introducing genetic material across the plasma membrane and then the nuclear membrane of target cells (Table 3). The majority of published literature is focused on a three distinct areas of investigation: replacing a malfunctioning gene, splicing redirection and gene silencing.

In the first strategy aiming at replacing a malfunctioning gene with a functional one, plasmid DNA containing the gene of interest is introduced into the nucleus of cells and expressed at levels that enable the improvement of the disease phenotype. KALA (Wyman *et al.*, 1997), MPG (Morris *et al.*, 1999) and POD (Johnson *et al.*, 2008) peptides are able to deliver plasmid DNA and enhance transgene expression both *in vitro* and *in vivo*. However, the bottleneck of this strategy is the relatively poor delivery efficiency, due to the fact that most of the unmodified CPPs do not enable efficient plasmid DNA condensation and, after internalization, they are unable to escape from the endosomes, remaining biologically unavailable (Lehto *et al.*, 2012). Recent studies report improved *in vivo* delivery of plasmid DNA following CPP modification by PEGylation (Read *et al.*, 2010) or stearylation (Lehto *et al.*, 2011).

A second strategy explored using CPPs is the correction of aberrant splicing patterns responsible for a variety of genetic disorders. Several CPPs have been successfully used for the delivery of antisense oligonucleotides or neutral oligonucleotide analogs, such as peptide nucleic acids (PNAs) and phosphorodiamidate morphorodiamidate morpholino oligomers (PMOs). By binding to complementary RNA target sequences, PMOs and PNAs act through steric blockage of the complementary RNA sequences, altering pre-mRNA splicing or inhibiting mRNA translation (Figure 3C). As an example, the correction of mutant dystrophin transcripts has been achieved in mouse model of muscular dystrophy by delivering PMOs through an analog of 6-aminohexanoic-spaced oligoarginine (R-Ahx-R)₄-Ahx-β-Ala (Moulton *et al.*, 2007). Similar correction efficiency levels have also been reported after *in vivo* delivery of PNAs targeting the exon 23 mutation in the dystrophin gene through Pip peptides (Ivanova *et al.*, 2008).

In a third approach, CPPs have been employed to mediate the delivery of small-interfering RNAs (siRNAs) for silencing gene expression. The RNA interference process is

mediated by short double-stranded RNAs which are fully complementary to their target sequence. When associated with the RNA-induced silencing complex (RISC), siRNAs hybridize to the target mRNA and induce the gene silencing by degrading it (Figure 3D). The first proof that CPPs are applicable for siRNA delivery and induction of effective gene silencing was provided in 2003 through a study based on noncovalent siRNA-MPG peptide complexes (Simeoni *et al.*, 2003). Few years later, an optimized version of MPG peptide has been used to deliver siRNA targeting cyclin B1, inducing downregulation of both protein and mRNA levels *in vitro*, and abolishing tumor growth in mice after systemic administration (Crombez *et al.*, 2009). In addition, an optimized endosomal escaping version of TP10 peptide (PF6 peptide) has been used to deliver siRNAs and induce significant *hprt1* gene knockdown in several tissues following systemic administration in mice (Andaloussi *et al.*, 2011).

Table 3. Examples of CPP-mediated nucleic acid delivery (Lehto *et al.*, 2012).

(a) Plasmid DNA			
CPP	Gene	Effect	
KALA	Luc	Luciferase expression	
MPG	Luc and cdc25C	Luciferase expression and cell cycle arrest	
Stearyl-R8	Luc	Luciferase expression	
Macro-branched Tat	Luc	Luciferase expression	
C-5H-Tat-5H-C	Luc	Luciferase expression <i>in vitro</i> and <i>in vivo</i>	
POD	RFP	RFP expression	
PEG-POD	Luc	Luciferase and β -galactosidase expression <i>in vitro</i> and <i>in vivo</i>	
PEG-POD	GDNF	GDNF expression <i>in vitro</i> and retinal degeneration rescue <i>in vivo</i>	
Stearyl-(RxR) ₄	Luc and EGFP	Luciferase and EGFP expression	
Stearyl-TP10	Luc and EGFP	Luciferase and EGFP expression <i>in vitro</i> and luciferase expression <i>in vivo</i>	
Stearyl-NLS-R8	Luc	Luciferase expression	
(b) Splice-correcting oligonucleotides			
CPP	ON type	Targeted mRNA	Model
Tat	PMO	Luciferase	HeLa pLuc 705
MAP	PNA	Luciferase	HeLa pLuc 705
Tat, TP, Pen	PNA	Luciferase	HeLa pLuc 705
M918	PNA	Luciferase	HeLa pLuc 705
(RxR) ₄	PMO	Dystrophin	DMD canine myoblasts, DMD mouse model
Pip peptides	PNA	Luciferase	HeLa pLuc 705,
		Dystrophin	DMD mouse model
R6-Pen	PNA	Luciferase	HeLa pLuc 705
B-peptide	PMO	Dystrophin	DMD mouse model
B-MSP	PMO	Dystrophin	DMD mouse model
P005	PMO	β -Globin	β -Thalassemia mouse model
(c) Small-interfering RNAs			
CPP	Target mRNA	Effect	
MPG Δ ^{NLS}	Luc/GAPDH	Luciferase and GAPDH downregulation <i>in vitro</i>	
MPG8/Chol-MPG-8	Cyclin B1	Inhibition of the cell proliferation <i>in vitro</i> /tumor reduction in mice	
CADY	GAPDH	GAPDH downregulation	
Stearyl-R8	GFP, MAP2b	GFP and MAP2b downregulation in primary cells	
Chol-R9	VEGF	VEGF downregulation and tumor reduction in mice	
Tat-DRBD	dGFP, dRFP, Luc, GAPDH, Oct-4	Efficient gene silencing in difficult-to-transfect primary and suspension cells and in mice luciferase model	
Tat-DRBD	EGFR, Akt2	EGFR and Akt2 downregulation induced tumor reduction and increased survival in mice	
PepFect6	Luc, EGFP, HPRT1, Oct-4, GAPDH	Efficient gene silencing in difficult-to-transfect primary and suspension cells and in mice upon systemic administration in two <i>in vivo</i> models	

Akt2: RAC-beta serine/threonine-protein kinase; Chol: Cholesteryl moiety; dGFP: destabilized green fluorescent protein; DMD: Duchenne muscular dystrophy; DRBD: Double-stranded RNA-binding domain; EGFP: Enhanced green fluorescent protein; EGFR: Epidermal growth factor receptor; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GDNF: Glial cell line-derived neurotrophic factor; HeLa pLuc 705: Model for splice correction efficiency; HPRT1: Hypoxanthine phosphoribosyltransferase 1; Luc: Luciferase; MAP2b: Microtubule-associated protein 2b; NLS: Nuclear localization signal; Oct-4: Octamer-binding transcription factor 4; PEG: Polyethyleneglycol; PMO: Phosphorodiamidate morpholino oligonucleotide; PNA: Peptide nucleic acid; POD: Peptide for ocular delivery; RFP: Red fluorescent protein; VEGF: Vascular endothelial growth factor.

4.4.4. Protein delivery

CPP-mediated delivery of functional proteins or peptides has been explored both for studying cellular processes and for developing novel and potentially specific therapeutic macromolecules. The first proof of concept of *in vivo* CPP-mediated protein delivery was provided by Dowdy and colleagues in 1999, showing that Tat- β galactosidase fusion protein could be delivered into almost all tissues including the brain, following intra-peritoneal injection into mice (Schwarze *et al.*, 1999). Since then, a growing list of transducible proteins covering a wide range of sizes and functional classes has been efficiently delivered by CPPs, and some examples are listed in Table 4.

A large body of literature is mainly focused on the exploration of CPPs as therapeutic carriers for peptides/proteins with the purpose to discover new drug programs. Generally, these strategies are based on the specific transfer of either inhibitory peptides directed against identified intracellular targets or functional proteins conceived to restore the intracellular level of downregulated endogenous proteins and to replace malfunctioning ones.

Studies using CPP-conjugated protein cargoes in oncology models have been reported. The tumor suppressor p53 has constituted a choice target as it is often nonfunctional in cancer cells. Different p53-derived peptides covalently linked to CPPs have been demonstrated to efficiently restore p53 functions in tumor cells. For example, polyarginine conjugated to p53 inhibits the proliferation of human glioma cells *in vitro* when the p53 protein is mutated to be ubiquitination resistant (Michiue *et al.*, 2005). Tat-mediated delivery of a peptide deriving from C-terminus of p53 induces the reduction of tumor growth upon intra-peritoneal injection into mice with B-cell lymphoma (Snyder *et al.*, 2004; Tang *et al.*, 2007). Protein domains deriving from natural protein inhibitors (p16^{Ink}, p21, p15 or p27^{kip}) of cyclin-dependent kinases have been also used for blocking cancer cell proliferation. *In vivo* tumor suppressor function has been reported for p27^{kip}-Tat fusion protein (Nagahara *et al.*, 1998) and for p16^{Ink}-derived peptide associated to penetratin (Hosotani *et al.*, 2002).

Table 4. Examples of delivery of peptides and proteins by cell-penetrating peptides (Dietz *et al.*, 2004)

Application	CPP	Cargo	Effect
Antibody delivery	Tat	anti-tumor antibody FAB fragments	delivery into breast carcinoma cell line
Antigen presentation	Tat	tumor-associated antigen OVA	antigen presentation to CD4 T-cells, tumor regression in mice
Apoptosis	Penetratin	Bak BH3 domain peptide	apoptosis induction in HeLa cells
	Penetratin	eIF4E-binding protein and eIF4G derived peptides	apoptosis induction in MRC5 cells
	Penetratin	p53 suppressor	inhibition of apoptosis
	VP22	neuroamidase	apoptosis induction in HeLa cells
	R ₁₁	calpastatin	inhibition of calpain activation in neurons
	Tat	Bcl-X _L	protection in various models of cerebral infarct
	Penetratin	inhibitory domain of calpastatin	Inhibition of calpain activation; apoptosis inhibition in LCLC 103H cells
Cancer treatment	Penetratin	p53-derived peptide	binding to MDM-2, specific cytotoxicity in tumor cell lines
	Penetratin	CDK-2 inhibitory peptide	apoptosis induction in transformed cells
	Tat, Penetratin	p53	apoptosis induction in breast cancer cells
	VP22	papilloma virus antigen	increased CD8 ⁺ -mediated anti-tumor response
Cardioprotection	Tat	δPKC inhibitor and εPKC activator	synergistic ischemia; cardioprotection by both peptides
	Tat	BH4 domain of Bcl-X _L	decrease in damage after ischemia on excised hearts
Cell cycle	Penetratin	p14 ^{INK4a} protein (cdk4/6 inhib.)	G ₁ arrest in primary fibroblasts
	Tat	CDK-2	entry into S-phase in transfected cell line
	Tat	p16 ^{INK4a} protein (cdk4/6 inhibiting) dominant-negative CDK-2, E1A	prevention of pRB hypophosphorylation, G ₁ arrest
	Tat	p27 ^{KIP1} protein (CDK-2 inhibitor)	G ₁ arrest in Jurkat T cell migration in HepG2 carcinoma cells
Cytoprotection	Tat	HSF70	protection against thermal stress and hyperoxia in HSF ^{-/-} cells
Drug resistance	poly-lysine	methotrexate	growth inhibition of CHO cells
Molecular imaging	Tat	^{99m} Tc	quick accumulation in Jurkat cells, distribution in the body after intravenous application, useful imaging agent
Mutagenesis	Tat	Cre recombinase	Lox-P recombination in fibroblast and ES cells, primary splenocytes
Oxidative stress	Tat	Cu, Zn-SOD	protection of HeLa cells against paraquat (SO radical donor)
Oxidative stress	Tat, Arg ₉	human catalase	protection against paraquat and H ₂ O ₂ in PC12 and HeLa cells, penetration into skin
Protein uptake	Tat	β-galactosidase	enzymatic activity in all tissues, including the brain after IP injection
	Tat	β-Gal, HRP, RNase A, <i>Pseudomonas</i> exotoxin A	delivery <i>in vitro</i> , and into heart, liver spleen, lung, muscle <i>in vivo</i>
	poly-lysine	HRP	accumulation in mouse fibroblasts
	poly-lysine	HRP, human serum albumin	accumulation in mouse fibroblasts
Tracking of cells <i>in vivo</i>	Tat	superparamagnetic nanoparticles	internalization into hematopoietic and neural progenitors
Translocation studies	VP22	GFP	translocation into COS-7 cells and muscle bystander cells
	Penetratin	fluorescein	fluorescence in pure, unilamellar vesicles
	Pep-1	GFP, β-Gal, antibodies	delivery into fibroblasts and other cells
	pVEC	fluorophore	transduction into melanoma and different epithelial cells
	Tat	eGFP	fluorescence in HeLa, COS-1 and Jurkat cells <i>in vitro</i>
	Tat	Superoxide Dismutase (Cu,Zn-SOD)	increased viability of paraquat-treated HeLa cells
	Tat, Penetratin, R ₇	fluorophore	transduction observed in non-fixed cells
Transportan	GFP, avidin, biotin-streptavidin-Texas red, antibody	transduction into BRL or COS-7 cells	

Other successful applications have been achieved conjugating CPPs to antiapoptotic peptides or proteins. Tat₄₇₋₅₇ conjugated to a peptide inhibitor of protein kinase C (now in clinical development as KAI-9803 by KAI Pharmaceuticals) significantly reduces apoptotic cell death and decreases the infarct size in rat model when administered either by intra-arterial or intraperitoneal injection (Bright *et al.*, 2004). Protection against apoptotic cell death has been also obtained by Tat₄₈₋₅₇-mediated delivery of D-JNKI1, a peptide inhibitor of c-Jun-N-terminal kinase in *in vitro* and *in vivo* models of cerebral ischemia (Hirt *et al.*, 2004). This conjugate product is now in clinical development as XG-102 by Xigen.

Pharmacological applications of CPP-conjugated antigens or antibodies have been also explored. As an example, Tat peptide has been used for the delivery of modular antigen molecules useful for treatment of allergy and vaccine production (Rhyner *et al.*, 2007). The same peptide has been shown to promote cellular internalization and nuclear import of a radiolabeled antibody directed against the intracellular cyclin-dependent kinase inhibitor p21^{WAF-1/Cip-1}, blocking breast cancer cells in G₁-S phase (Hu *et al.*, 2007).

The potency and efficacy of CPPs has been also validated for treatment of inflammatory response. For example, Tat-derived PTD4 peptide inhibits the activity of the transcription factors linked to allergic diseases was achieved using (McCusker *et al.*, 2007). Inhibition of inflammatory response by reduction of cytokine secretion has been also shown using a fusion protein composed of Tat-peptide and dominant negative forms of Ras (Myou *et al.*, 2003).

All these studies demonstrate the feasibility of using CPPs as carriers for cell-impermeable molecules with intracellular targets (Chen *et al.*, 2007). The therapeutic potential of CPPs in drug development has led to the formation of industrial companies with proprietary cell-penetrating technologies (Table 5). The growing amount of *in vivo* applications and the successful results obtained for CPP-based strategies under clinical development confirm the utility of CPPs as biomedical tools and molecular therapeutics.

Table 5. CPP-conjugated compounds in clinical development of (Johnson *et al.*, 2011; Schmidt, 2013)

Company	Compound	Target/Action	CPP	Indication	Status
Capstone Therapeutics	AZX100	HSP20	PTD4	Dermal scarring	Phase 2
KAI Pharmaceuticals	KAI-9803	Protein kinase C δ inhibitor	Tat PTD	Myocardial infarction and cerebral ischemia	Phase 2b
	KAI-1678	Protein kinase C ϵ inhibitor	Tat PTD	Pain	Phase 2a
	KAI-1455	Protein kinase C ϵ activator	Tat PTD	Cytoprotection/ischemia	Phase 1
Xigen	XG-102	c-Jun-N-terminal kinases	Tat PTD	Hearing loss	Phase 2b
				Stroke	Phase 1
Revance Therapeutics	RT001	Transdermal delivery of Botulinum toxin type A	Tat PTD	Wrinkles	Phase 2b
				Excessive sweating	Phase 1
CellGate	PsorBan	Transdermal delivery of cyclosporin A	R8	Psoriasis	Phase 2 discontinued
AVI Biopharma	AVI-5126	Inhibition <i>c-myc</i> gene	(RAHxR) ₄	Cardiovascular restenosis	Phase 2
CDG Therapeutics	P28	Inhibition of p53 ubiquitination	p28 peptide	Cancer	Phase 1

4.5. CPPs: current limits and improvements

CPPs have effectively opened the door for cell-impermeable compounds directed against intracellular targets. Classes of compounds, that were inconceivable as therapeutic candidates in the past, are now worthy of consideration for drug development (Chen *et al.*, 2007). Except for CPP-based drugs that are currently in clinical development (Table 5), some issues regarding their *in vivo* applications still remain to be solved.

While the noninvasive mechanism of CPP cellular uptake provides a valuable means of intracellular delivery, the non-specificity creates a significant challenge in targeted delivery to the disease site by systemic administration (MacEwan *et al.*, 2013). In addition to the lack of cell specificity, the metabolic instability of peptides in presence of serum, the rapid clearance and the immunogenicity are other factors that can negatively affect the *in vivo* CPP efficacy (Chen *et al.*, 2007). To solve these problems, a number of approaches are being actively

investigated. For instance, penetratin, Tat and MTS peptides have been conjugated to elastin-like thermally sensitive polypeptides (ELPs) (Bidwell *et al.*, 2010). Under physiological conditions (37 °C), ELPs are likely to be cleared. However, when tumor cells are externally heated (40-42 °C) ELPs aggregate and selectively accumulate in tumors, mediating peptide escape from the tumor vasculature and its specific entry into tumor cells (Bidwell *et al.*, 2010). To avoid that CPPs are internalized into “wrong” cells, recent studies are focused on the engineering of novel peptides starting from known “homing” sequences (Alberici *et al.*, 2013) or on the screening tumor lineage-homing CPPs from random libraries (Kondo *et al.*, 2012).

Regarding the issues of peptide degradation, a method to improve the circulation time is to shield CPPs with hydrophilic polymers, such as polyethylene glycol (PEG) that can provide a protective corona over CPP carriers (MacEwan *et al.*, 2013). Alternatively, D-isomers of some CPPs are as effective as L-isomers in cell penetration, suggesting that this modification is likely sufficient to stabilize CPPs in plasma without having an impact on cell-penetrating activity (Vives *et al.*, 2008). With the purpose of both increase peptide half-life upon administration and control the drug release into tumor cells, the combination of liposomal carriers, PEG chains, Tat peptide and a cancer cell-specific antibody has been recently described (Koren *et al.*, 2012a). At normal pH, the steric hindrances created by the PEG shield the surface-attached Tat peptides. Upon the exposure to low pH (typical environment of solid tumors), this multifunctional carrier exposes Tat moieties after the degradation and removal of PEG chains (Koren *et al.*, 2012a). The presence of the antibody defines the interaction with tumor cells but not normal cells and Tat peptides enhance cellular uptake.

The knowledge of eventual immunogenic and immunotoxic effects induced by CPPs is of particular interest for therapeutic applications (Chen *et al.*, 2007). It is unlikely that CPPs with a short plasma half-life and rapid tissue distribution could generate an immune response by intravenous administration (Chen *et al.*, 2007). However, the administration route of CPP-conjugates may have different effects on the immune system, *i. e.* activating directly the lymphatic system upon a subcutaneous administration (Porter *et al.*, 2001). A study performed on long-term *in vivo* administration of penetratin peptide and its analog (PenetraMax) has shown that both peptides are not toxic either systemically or locally, and any significant difference in the release of inflammatory and immunogenicity mediators in

plasma has been observed after nasal administration (Khafagy el *et al.*, 2013). In addition, Tat peptide, stearyl-(RxR)₄, TP10 and its PF analog peptides do not induce any increase of cytokine levels in serum and do not evidence undesired toxicity and inflammation after intravenous injections in mice (Suhorutsenko *et al.*, 2011). The long-term toxicity of these peptides still has to be evaluated.

5. The Epstein-Barr virus ZEBRA transcriptional factor

Analogously to other transcription factors such as Antennapedia or Tat, the Epstein-Barr virus ZEBRA transcriptional activator possesses cell-penetrating property (Mahot *et al.*, 2005). Originally named EB1 (Chevallier-Greco *et al.*, 1986) and later called Zta, BZLF1 or ZEBRA (Countryman *et al.*, 1987; Lieberman *et al.*, 1990), this protein is mainly known to activate the switch between the latent and replicative life cycle of the Epstein-Barr virus (EBV). Mahot *et al.* have demonstrated that, when exogenously added to cell medium, full-length ZEBRA protein enters lymphoid cells (both EBV-positive and EBV-negative cells) and accumulates into the nucleus in a time-dependent manner (Mahot *et al.*, 2005). They also observed that after internalization, exogenous protein does not induce the EBV re-activation (Mahot *et al.*, 2005).

Epstein-Barr virus

EBV is a human herpesvirus that infects over 90% of the world population. The primary infection is generally silent but can be clinically revealed by a benign disease called infectious mononucleosis. After primary infection, the viral genome persists as a lifelong, asymptomatic infection of B cells. Despite its benign appearance, EBV has growth-transforming ability and is etiologically linked to different B cell malignancies, including Hodgkin's disease, Burkitt's lymphoma, nasopharyngeal carcinoma (Young et al., 2004). EBV periodically reactivates and replicates in a lytic manner in a subset of B cells - a form of infection essential for viral propagation and transmission (Amon et al., 2005). Disruption of viral latency in EBV-infected B cells is determined by an intricate cascade of events initiating at the plasma membrane. Activation of the lytic cycle can be induced by a variety of reagents, including anti-immunoglobulin, calcium ionophore, butyrate and the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (Speck et al., 1997). The first EBV genes expressed during the lytic infection are the immediate-early genes BZLF1 and BRLF1 encoding for ZEBRA and Rta transcription factors respectively, and both factors are necessary to activate the switch (Chevallier-Greco et al., 1986). Afterwards, ZEBRA activates the promoters of EBV lytic genes by binding target sites termed ZEBRA response elements (ZREs), inducing a cascade of expression of over 50 viral genes.

ZEBRA is a 245 residue protein that belongs to the bZIP family of transcription factors. This protein is composed of a N-terminal transactivation domain (TAD, residues 1-140), a basic DNA-binding domain (DBD, residues 175-195), a coiled-coil dimerization region (DIM, residues 196-220) and a C-terminal tail (Figure 4A). Proteins belonging to bZIP family (*e. g.* yeast GCN4 and human c-Fos and c-Jun) are homo- or hetero- dimers that contain a bZIP domain composed of highly basic DNA-binding regions adjacent to regions of α -helix that fold together as coiled coils (leucine zipper) (Sinclair, 2006). Their bZIP-mediated interaction with DNA is dependent on dimer formation (Sinclair, 2006). However, the bZIP domain of ZEBRA protein is unusual. Unlike the other leucine zipper proteins, ZEBRA bZIP domain (approximately residues 175-220) lacks the characteristic heptad repeat of leucine residues that ordinarily mediates dimerization (Chang *et al.*, 1990; Petosa *et al.*, 2006). In addition, ZEBRA bZIP domain is insufficient to form a stable homodimer at physiological temperature or to bind DNA with high affinity (Hicks *et al.*, 2003; Petosa *et al.*, 2006). The C-terminal part of ZEBRA (residues 175-237) has been recently crystallized in its DNA-bound form (Petosa *et al.*, 2006). A continuous stretch of α -helix that encompasses both the basic and the DIM region (to Met 221) was identified, as expected for a bZIP protein. However, a hairpin turn was observed at the end of the α -helix, which results in an orientation of the C-terminal region antiparallel to the ZIP domain (Figure 4B). This C-terminal tail is composed of a one-turn helix (helix α C) followed by an extended stretch of residues, which intercalate with the ZIP region resulting in intra- and inter- molecular hydrophobic interactions (Petosa *et al.*, 2006; Sinclair, 2006). The interaction of C-terminal tail with the coiled coil greatly enlarges the dimer interface and increases its stability despite the missing of the characteristic heptad repeat leucine zipper motif (Petosa *et al.*, 2006).

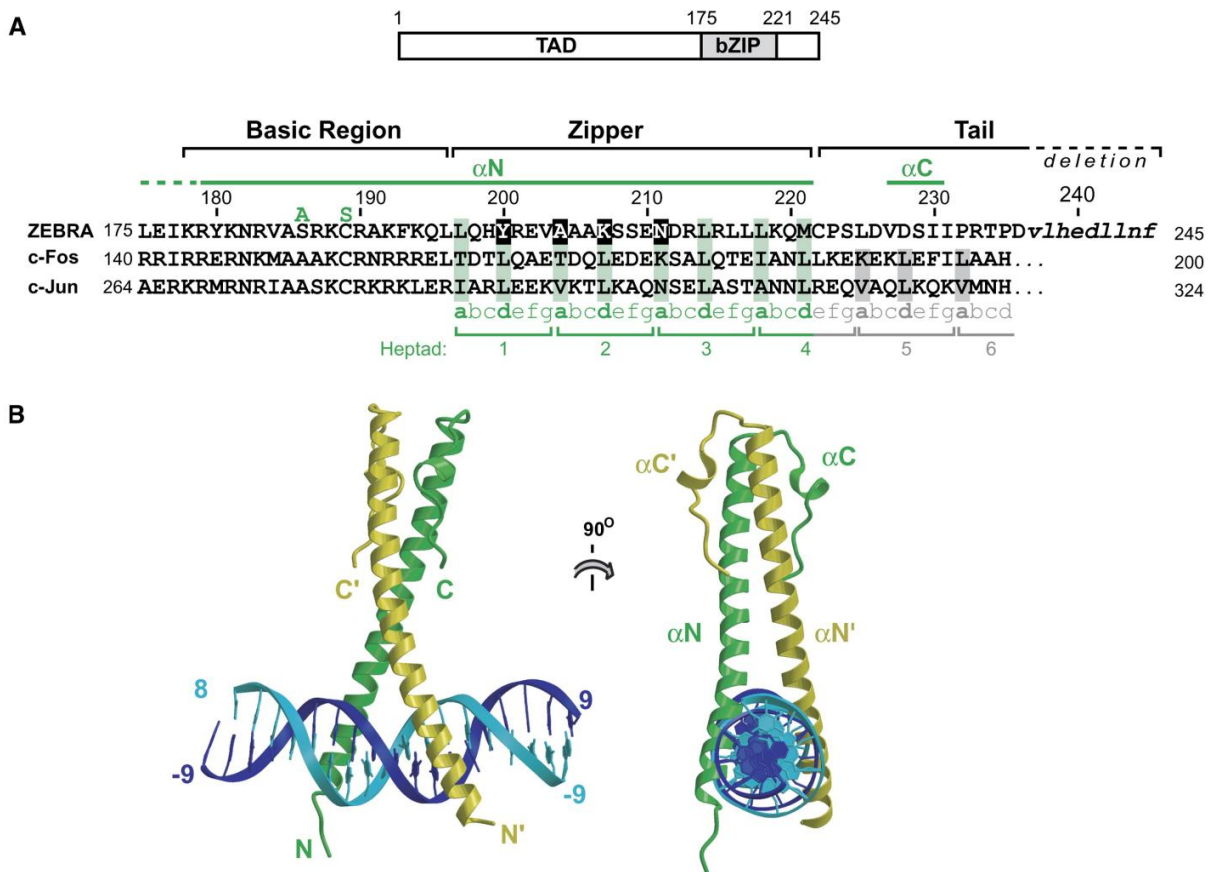


Figure 4. Structure of ZEBRA protein. A) Schematic representation of protein sequence. ZEBRA is composed of N-terminal trans-activation domain (TAD), a bZIP region (expanded below) that contains the basic and the dimerization domains, and a C-terminal tail. B) Orthogonal views of the ZEBRA C-terminal part-DNA complex. The coiled coil bends towards one end of the DNA, with one bZIP helix curved (yellow) and the other essentially straight (Petosa *et al.*, 2006).

5.1. MD: ZEBRA-deriving CPP

The molecular dissection of the ZEBRA protein has been initiated few years ago in our laboratory, with the aim to identify the amino acid region responsible for its cell-penetrating property. Different truncations covering the entire amino acid sequence of the full-length protein have been constructed and tested for their ability to translocate into cells when fused to the reporter protein eGFP (Rothe *et al.*, 2008; Rothe *et al.*, 2010). The analysis of these truncated forms has revealed that the region responsible for cellular penetration is located in C-terminal part of ZEBRA protein in correspondence of bZIP domain. In particular, the minimal amino acid sequence (MD) required for internalization is composed of 54 residues (residues 170-220 of full-length ZEBRA protein) and contains both the basic DNA-binding domain (DBD) and the coiled-coil dimerization region (DIM) (Rothe *et al.*, 2010). The MD

cell-penetrating peptide efficiently translocates reporter proteins of different size, such as eGFP (30 kDa) and β -galactosidase (116kDa) into several normal and tumor cell lines. The cargo molecules remain functional after internalization and no toxicity is associated to this process.

The MD entry mechanism has been investigated by using different experimental approaches, *i. e.* by measuring the uptake efficiency in energy depletion conditions, at low temperatures, in presence of drugs that selectively inhibit different internalization pathways; and by evaluating through confocal microscopy the peptide-cargo co-localization with molecular markers of known internalization pathways. The lack of uptake inhibition in presence of endocytosis inhibitors and the absence of co-localization with endosomal markers have demonstrated that MD-cargo proteins are internalized via direct translocation across the lipid bilayer (Rothe *et al.*, 2010). In addition, the electrostatic interaction with heparan sulfate proteoglycans is required for peptide entry. In fact, the MD basic domain (DBD) is believed to mediate the initial binding with cell surface and the DIM domain is supposed to destabilize the lipid bilayer through hydrophobic interactions (Rothe *et al.*, 2010). As its uptake is largely independent from endocytosis, MD translocates cargo proteins into cells circumventing their endosomal entrapment, which is the main limit for most known CPPs. This aspect represents a major strength of this peptide, notably for its application as delivery system for therapeutic uses.

As mentioned above, full-length ZEBRA protein is a transcription factor which binds DNA through its bZIP domain. With the prospect to use the ZEBRA-deriving CPP as delivery system, its DNA-binding capacity has been investigated. Interestingly, the fusion at MD C-terminus of cargo proteins induces a loss of its DNA-binding capacity (Rothe *et al.*, 2010). According to crystal structure, DNA binding of the ZEBRA protein requires complex conformation of its C-terminal part, and mutations in the latter inactivate transcription factor activity (Petosa *et al.*, 2006). The conformational changes, which occur upon fusion with cargo molecules, compromise the MD DNA-binding property. The loss of this property represents a tremendous advantage for the development of ZEBRA-deriving CPP as delivery system.

6. Aim of the study

The aim of this thesis is to explore the cell-penetrating properties of the MD peptide deriving from the EBV ZEBRA protein and evaluate its application as protein delivery system. The project is structured into three major sections:

- (i) Optimization of the MD-CPP by size reduction and evaluation of the amino acid composition role in the translocation process across the cell membrane;
- (ii) Design of an antitumoral MD-mediated protein therapy based on the vectorization of the translational factor eIF3f;
- (iii) Investigation of MD cell-penetrating properties in pathogenic fungus *Candida albicans* cells.

CHAPTER I

Optimization of the ZEBRA cell-penetrating peptide

Roberta Marchione, Lavinia Liguori, David Laurin and Jean-Luc Lenormand, ZEBRA cell-penetrating peptide penetrates cells by an endocytosis-independent mechanism, article in preparation.

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Preface

The first dissection study of EBV ZEBRA transcriptional factor performed in our laboratory, has allowed to identify the amino acid region involved in cellular internalization (MD, amino acids 170-220) and to characterize its direct penetration mechanism in mammalian cells. The objective of the study described in this chapter was to optimize by further size reduction the MD sequence without modifying the internalization mechanism, and evaluate the role of its amino acid composition in the translocation process across cell membrane. The identification of a shorter MD peptide able to enter cells by endocytosis-independent mechanism is suitable for efficient drug delivery strategies. Indeed, the direct addressing of hydrophilic cargoes to intracellular compartments represents a great advantage for therapeutic applications as the cargo molecules can be directly delivered into cells without being entrapped and degraded in endosomes/lysosomes, and thus preserving their biological activity.

The strategy employed for studying the cellular uptake of MD truncation mutants is based on a method already validated in our laboratory (Rothe *et al.*, 2008), which consists in: (i) the generation of fusion proteins containing the different cell-penetrating sequences fused to the N-terminus of a reporter green fluorescent protein (eGFP), (ii) the production of the recombinant proteins in *Escherichia coli* expression system, and (iii) the purification of the fusion proteins by affinity chromatography. As the internalization mechanism is influenced by the presence and the size of the cargo molecule, eGFP has been chosen as a model protein cargo taking into account its molecular weight (30kDa) and the possibility to directly monitor the internalization process.

The ZEBRA MD is composed of a positively charged domain (DBD domain) and a hydrophobic leucin-rich domain (DIM domain). A first MD truncation mutant has been generated by removing eight amino acids from its N-terminal end (MD₁₁). The MD₁₁ peptide maintains the same translocation efficiency and mechanism reported for MD.

For the designing of further truncated forms, we decided to delete amino acids from MD₁₁ C-terminal end for two main reasons. Firstly, we noticed that when produced in *E. coli*, some fusion proteins were not expressed in soluble form. This effect can be ascribed to an

incorrect three-dimensional protein folding, probably due to the presence of hydrophobic amino acids in the C-terminal part of MD peptide. Secondly, some recombinant proteins showed a tendency to aggregate even after purification. Thus, we supposed that the deletion of the C-terminal residues could minimize the hydrophobic interactions of MD peptide with fused cargo proteins, and probably increase the solubility of the fusion proteins. Therefore, we decided to investigate the role of the C-terminal part of MD peptide in uptake process by generating a series of iterative truncation mutants.

The human cervical cancer HeLa cells were chosen as target cells for studying the internalization process of the MD₁₁-derived truncations. The deletion of C-terminal residues impaired the cellular penetration efficiency. When we investigated the effect of these deletions on the uptake mechanism, we discovered that the shortening and the removal of the dimerization domain in the CPP sequence induced a modification in the molecular entry mechanism from a direct translocation to endocytosis. Even though we cannot exclude that different entry routes can be simultaneously used, the main uptake mechanism of MD₁₁-eGFP fusion proteins is endocytosis-independent. Therefore MD₁₁ represents a promising carrier for delivering biologically active macromolecules with therapeutic potential.

In conclusion, this study has allowed to (i) find an eight amino acid shorter MD cell-penetrating peptide (MD₁₁) able to translocate HeLa cells with 100% efficiency through an endocytosis-independent mechanism, (ii) clarify the role of the DIM domain in the uptake process and (iii) demonstrate that this amino acid region is essential for both peptide entry efficiency and its endosomal escape.

At the light of these results, it becomes evident that approaches, other than peptide hydrophobicity reduction, should be used to overcome some issues related to recombinant fusion protein solubility or aggregation.

ZEBRA cell-penetrating peptide penetrates cells by an endocytosis-independent mechanism

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Abbreviations: Cell-penetrating peptides (CPPs), minimal domain (MD), Epstein-Barr virus (EBV), dimerization domain (DIM), DNA-binding domain (DBD), enhanced green fluorescent protein (eGFP), Dulbecco's modified Eagle's medium (DMEM), Dulbecco's Phosphate-Buffered Saline (DPBS), methyl- β -cyclo-dextrin (M β CD), Fluorescence-activated cell sorting (FACS), Confocal scanning laser microscopy (CSLM), phosphatidylcholine (PC), sphingomyelin (SM), cholesterol (Chol)

1. Abstract

Background: Cell-penetrating peptides (CPPs) represent a noninvasive method for delivering functional biomolecules into living cells. We have recently shown that the Epstein-Barr virus transcriptional factor ZEBRA contains a protein transduction domain, named Z9 or minimal domain (MD). Only few of currently identified CPPs including MD are able to rapidly cross the mammalian cell membrane without being entrapped into endosomal compartments, even when fused to cargo macromolecules. In this work, we reduced the size of the transduction domain without modifying its internalization mechanism.

Methods: A series of MD deletion mutants has been engineered and their cellular uptake was analyzed by confocal microscopy and FACS.

Results: We identified a domain MD₁₁ (8 amino acids shorter than MD) able to enter mammalian cells with 100% efficiency via direct translocation. All the generated truncated forms exhibited reduced cellular uptake and penetrated into cells through endocytic mechanisms.

Conclusions: These results have highlighted the role of the MD₁₁ C-terminal region as essential for efficient cellular entry and endosomal escape.

General significance: This endosomal escape peptide could be used either to engineer novel CPPs or as a carrier for delivering biologically active macromolecules with therapeutic potential.

2. Introduction

Many therapeutic targets have been found located within cells but the effective transport of hydrophilic active molecules such as proteins or peptides across the cellular plasma membrane has represented a serious obstacle for many decades. A promising approach that seems to be the solution for overcoming the cellular barrier, has emerged with the discovery of the cell-penetrating peptides (CPPs), also referred to as protein transduction domains. Generally, CPPs are defined as relative short peptides with the ability to gain access to the cell interior and promote the intracellular delivery of conjugated cargoes [1]. Since the discovery of the first CPP from the HIV Tat protein [2, 3], a variety of transducing peptides has been identified, including both naturally occurring domains and synthetically derived sequences. Well known examples include the penetratin peptide [4], VP22 [5], pVEC [6],

polyArginine [7], Transportan [8], etc. The common feature of CPPs is their typically high content in basic arginine and lysine residues, leading to a positive net charge of the peptides, which is considered to be crucial for initial membrane interaction through binding to negatively charged phospholipids and glycosaminoglycans [9]. The number of applications using CPPs is increasing, and so far more than 300 studies from *in vitro* to *in vivo* have been reported [10]. Indeed, the interest for CPPs is mainly due to their low cytotoxicity and to the fact that there is no limitation for the type of cargo. CPPs have been used to improve delivery of cargo that varies greatly in size and nature, including small molecules, oligonucleotides, plasmid DNA, peptides, proteins, nanoparticles, virus and lipid-based formulations [11]. Despite the similarities among CPPs, the translocation mechanisms may vary considerably. The two main pathways suggested for cellular uptake are direct penetration and endocytosis [12]. However, the impact of these two mechanisms on the biological function of the transported cargo is different. Contrary to direct penetration, during endocytosis, the cargo can be entrapped and consecutively partially degraded into endosomal compartments, thus leading to the loss of its activity. This aspect represents the main limitation towards the therapeutic use of CPPs as delivery systems for biologically active drugs. When conjugated to cargo molecules, the cellular uptake of most widely used CPPs such as penetratin, Tat, pVEC and transportan is shown to proceed through an endocytic pathway [13-15]. In a previous study, we identified a novel cell-penetrating peptide able to cross the cell membranes in an endocytosis-independent mechanism even when fused to cargoes, as shown with eGFP and β -galactosidase reporter proteins [16]. This CPP derives from the Epstein-Barr virus (EBV) ZEBRA transcription factor. A reductionist study of full-length ZEBRA protein has allowed to identify the amino acid region (named as Minimal Domain, MD) implicated in cellular uptake [16]. The region required for internalization spans residues 170-220 from the ZEBRA protein and contains two contiguous domains: a positively charged domain (DNA-binding domain, DBD) and a hydrophobic leucin-rich domain (dimerization domain, DIM). The DBD is believed to mediate cell surface binding of the MD to the negatively charged heparan sulfate proteoglycans while the DIM domain facilitates translocation through the lipid bilayer by hydrophobic interactions [16].

In the present study, we aimed at reducing the size and the hydrophobicity of the transduction domain and at describing the amino acid sequence required for its cellular uptake. We produced MD truncations in fusion to the eGFP reporter protein and evaluated the

ability of these constructions to translocate through the membrane of HeLa cells. We identified a MD shorter peptide (MD₁₁) able to enter mammalian cells with 100% efficiency by an endocytosis-independent mechanism. Further trimming of the DIM domain from the MD₁₁ peptide led to a decrease in the translocation efficiency and to an alteration of the uptake mechanism. The results presented here reveal the role of the whole DIM domain appearing as necessary for endocytosis-independent cell internalization. This import mechanism is an attractive requisite for developing MD₁₁-mediated uptake of macromolecules in therapeutic applications and strengthens this delivery system compared to most others.

3. Materials and Methods

3.1. Cloning, expression and purification of the MD_x-eGFP fusion proteins

The cloning, the expression and the purification of the free eGFP and the recombinant fusion proteins MD_x-eGFP were performed as described in Rothe *et al.* [17]. Briefly, the DNA fragments encoding for the MD_x sequences were generated by PCR and ligated upstream of the 5'-end of eGFP gene into pET15b expression plasmid. All recombinant proteins were expressed in *E. coli* BL21(DE3) cells by inducing the expression at OD_{600nm} of 0.8 with 0.5mM IPTG for 18h at 16°C. The proteins of interest were recovered by sonication in 20mM Tris/HCl pH 7.4, 500mM NaCl, 10% glycerol, 2mM DTT, 10mM imidazole (5mL per gram wet pellet) supplemented with a complete protease inhibitor cocktail (Roche). The soluble fractions were purified onto nickel sepharose HisGraviTrap columns (GE Healthcare) by gravity-flow and eluted in the same buffer by a stepwise increase (at 100, 175, 250 and 500mM) of imidazole content. Proteins were separated onto a 15% SDS-polyacrylamide gel electrophoresis and analysed by Coomassie blue staining or by Western-blotting using an anti-His tag antibody HRP-coupled (Sigma, 1:10000 dilution). Prior to their use for cellular uptake experiments, purified eGFP and MD_x-eGFP proteins were dialyzed against PBS and 25mM Hepes/KOH pH7.0, 150mM NaCl and 10% glycerol respectively using a MWCO 8000 SpectraPorTM dialysis tubing (Spectrum Laboratories). The yield of the purified His-tagged proteins was quantified by BCA Protein Assay Kit according to the manufacturer's instructions (Pierce).

3.2. Cell culture

HeLa cells were maintained in DMEM (PAA, GE Healthcare) supplemented with 10% heat-inactivated fetal bovine serum (PAA, GE Healthcare), 100 units/mL penicillin and 50µg/mL streptomycin (Gibco). Cells were cultured at 37°C in a humidified 5% CO₂ atmosphere incubator.

3.3. Confocal microscopy

HeLa cells (5×10^4 cells/well) were seeded onto an 8-well Lab-Tek™ chambered coverglass (Nunc) in complete cell culture media 24h before treatment. After removal of the medium, the cell layers were rinsed twice with DPBS and subsequently exposed at 37°C for 4 hours to 0.3µM of recombinant proteins in a fresh serum-free medium. Thereafter, the incubation solutions were removed and the cells were washed three times with DPBS. Living cell preparations were observed with a LSM 710 confocal scanning laser microscope (Carl Zeiss, Jena, Germany), using a 63×, NA 1.2, C-apochromat water-immersion objective (Carl Zeiss). The experiment was carried out at 488nm excitation and fluorescence was collected with a 510-560nm filter. 16 successive optical slices were captured along the cell z axis, with a step of 1µm. For localization study, cells were also fixed for 15 minutes in 4% paraformaldehyde at room temperature and then stained with 10µM Nile Red. The images were acquired with 488nm for eGFP and 633nm for Nile Red excitation and fluorescence was collected between 502-541 and 580-668nm filters respectively.

3.4. Cellular uptake assays

For transduction experiments, 1.5×10^5 HeLa cells/well were cultured on 12-well plates until 70% of confluence. Cells were washed twice with DPBS and incubated with 0.3µM of recombinant proteins in fresh serum-free culture medium for 4 hours at 37°C. After incubation, cells were trypsinized with 0.5% trypsin/EDTA solution (PAA, GE Healthcare) for 10 min at 37°C to remove the extracellular bound proteins. The trypsin was then neutralized by adding complete medium and the cells were washed twice with DPBS.

For endocytic inhibition experiments, cells were first pre-incubated for 30 minutes with different endocytic inhibitors at the following concentrations: 50µg/mL nystatin, 100nM

wortmannin and 5mM methyl- β -cyclo-dextrin (Sigma). After pre-incubations with the endocytic inhibitors, the recombinant proteins were added to cell cultures in the presence of drugs and treated as indicated above.

Cell-associated fluorescence was detected using a FACS Canto II BD Biosciences flow cytometer. We used a 488nm laser for excitation and 530/30 bandpass filters for emission. The results are reported as the mean fluorescence intensity from living cells gate of 30000 events recorded and analyzed with the FACSDiva software.

3.5. Preparation and observation of lipid vesicles

Giant unilamellar vesicles (GUVs) were created by sucrose hydration method [18]. Three different lipid compositions were used in order to obtain fluid (100% PC), rigid (SM:Chol 50:50 % molar ratio) and semi-fluid (PC:SM:Chol 30:30:30 % molar ratio) GUVs. 50 μ g of lipid mixtures in chloroform were deposited in an 8-well Lab-TekTM chambered coverglasses and dried under nitrogen. The dried film was hydrated overnight at 4°C with 25mM Hepes pH7.4, 250mM sucrose. After re-hydration, 1 μ g of MD₁₁-eGFP was added to GUV solution. The lipid component was labeled with Nile Red dye (10 μ M), and CSLM images were acquired as described above.

4. Results

4.1. Design and expression of MD analogs

The ZEBRA MD protein transduction domain (amino acids 170-220) consists of 14 basic residues (Lys and Arg) and 15 hydrophobic residues, mainly located at the C-terminal dimerization domain (Fig. 1A) [16]. To better understand the specific contribution of each amino acid in the internalization process, we designed a series of iterative MD truncation mutants. A first deletion was realized by removing the first eight amino acid residues located upstream of the basic region at the N-terminus of the MD, resulting in a sequence named as MD₁₁ (Fig. 1A). In transduction experiments on HeLa cells, recombinant proteins containing MD₁₁ fused to the fluorescent reporter protein eGFP were able to penetrate with the same efficiency than MD (R Rothe and JL Lenormand, unpublished data). MD₁₁ represents the starting peptide for successive optimizations.

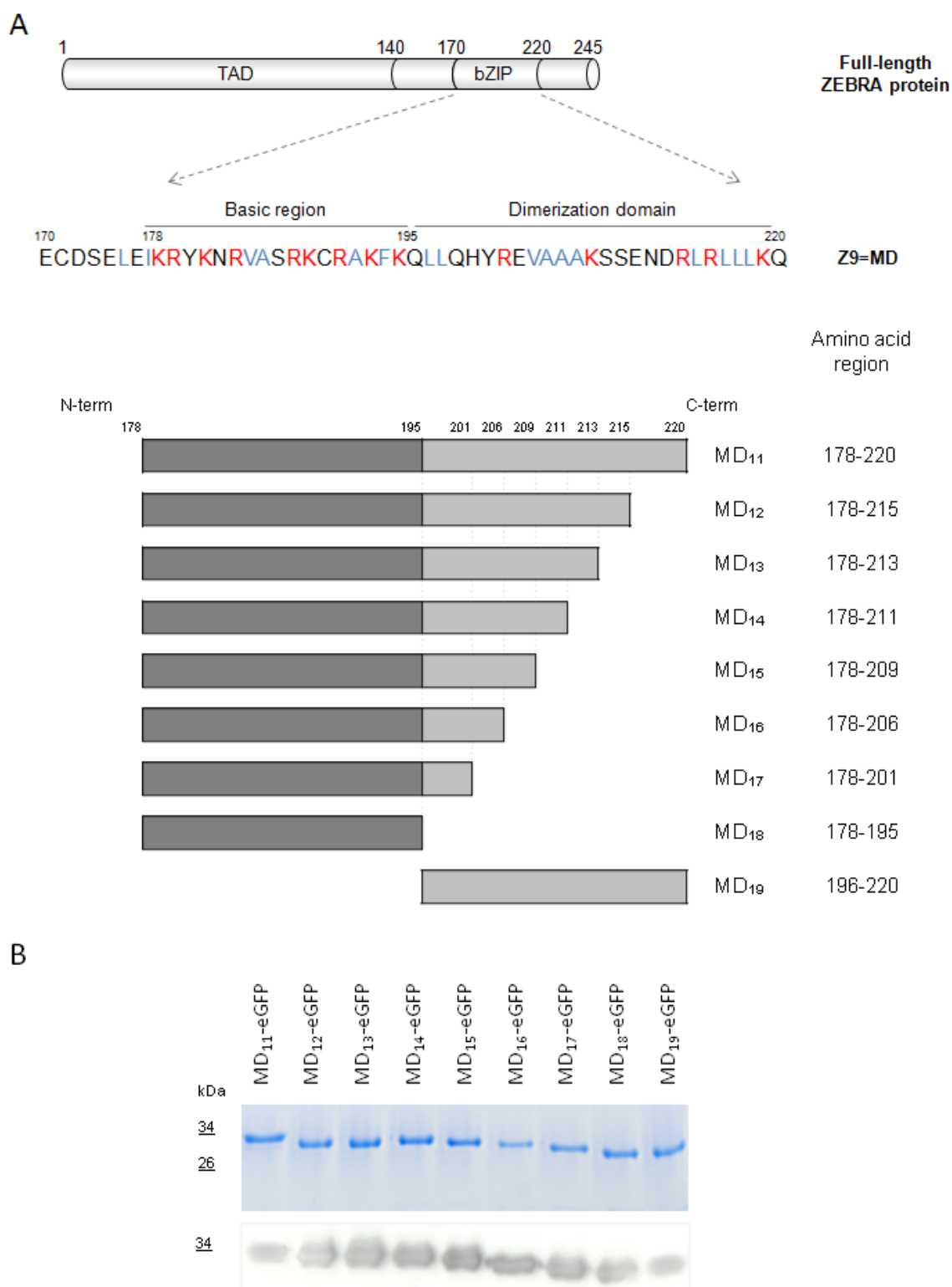


Figure 1. A) Amino-acid sequence of ZEBRA MD cell-penetrating peptide (aa 170-220) and scheme of the deletion mutants. Amino acids are designated with one-letter code and numbered according to the full-length ZEBRA protein sequence. Basic amino acids are shown in red, whereas hydrophobic amino acids in blue. B) Recombinant fusion proteins, containing each peptide fused to the N-term of eGFP, were separated on a 15% SDS-polyacrylamide gel and visualized by Coomassie Blue staining and detected by Western blotting using anti-His antibody.

In order to increase the MD₁₁ solubility, we further engineered truncations by removing from a minimum of five to a maximum of nineteen amino acids from the hydrophobic C-terminus (Fig. 1A). In addition, we produced two more truncations containing either only the basic (MD₁₈) or the hydrophobic (MD₁₉) region. We then expressed each peptide fused to the N-terminal end of eGFP. The his-tagged recombinant fusion proteins were produced using an *E. coli* expression system and purified by nickel affinity chromatography. The purity of the MD_x-eGFP proteins was analyzed by Coomassie blue staining and by western-blotting using an anti-his antibody (Fig. 1B). Recombinant proteins were pure at around 85%.

A crystallographic study has revealed that the DNA-bound ZEBRA protein (residues 175-245) is an extended bZIP helix [19]. With the use of the helical wheel projections, the sequences of the MD truncations were examined (Fig. 2). The wheels for all deletions have both a polar and a hydrophobic character. In each helix, there are two spatially organized regions of polar residues interrupted by a few hydrophobic residues. The exact content in polar and non-polar residues for each truncation is summarized in the Table 1. In all cases, except for MD₁₉, the peptides are net positively charged. The shortening and deletion of the DIM domain affect both charge and hydrophobicity contributions: the non-polar and negative amino acid content is reduced and the hydrophilicity (negative GRAVY value, [20]) of the peptides is increased.

4.2. Confocal microscopy

The transduction ability of MD deletions was investigated by confocal microscopy on living HeLa cells after 4 hours treatment with 0.3 μ M of each recombinant protein according to results reported in Rothe *et al.* [16]. The cellular uptake of the recombinant proteins was checked by direct visualization of the intracellular eGFP fluorescence. As expected, no fluorescence signal was detected on HeLa cells incubated with free eGFP (Fig. 3). Except for MD₁₉, eGFP was localized at the cell membranes and intracellularly in cells treated with the MD₁₁ and its truncated forms (Fig. 3). Then, to better delineate cell contours and evaluate the intracellular distribution of the recombinant proteins, cells were fixed and the lipid membranes stained with the fluorescent dye Nile Red. As shown in Fig. 4, Z-stack analysis of treated cells confirmed that most of the MD₁₁ analogs lacking of few residues at C-terminus still maintained the cell penetrating ability. However, as visible in both Fig. 3 and Fig. 4, the

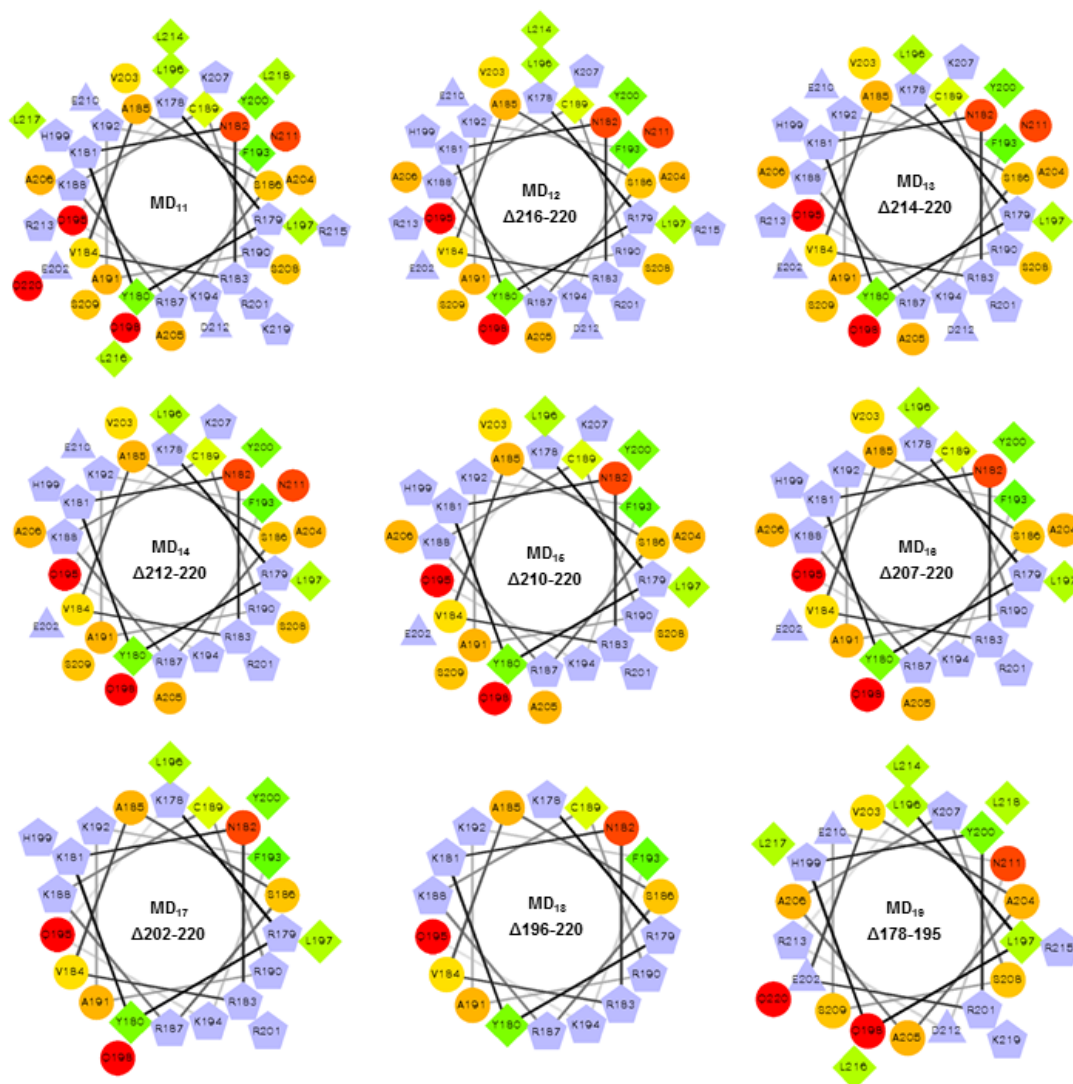


Table 1

Peptide	Number of residues	Net charge at pH 7	Positive charges K,R, H	Negative charges D, E	Aromatic residues Y,F	Polar residues (without net charge) N, Q, S	Non polar residues A, C, F, I, L, V, Y	Grand average of hydrophobicity (GRAVY)
MD ₁₁	43	11,5	15	3	3	8	17	-1.151
MD ₁₂	38	10,5	14	3	3	7	14	-1.408
MD ₁₃	36	9,5	13	3	3	7	13	-1.467
MD ₁₄	34	9,5	12	2	3	7	13	-1.318
MD ₁₅	32	10,5	12	1	3	6	13	-1.181
MD ₁₆	29	9,5	11	1	3	4	13	-1.114
MD ₁₇	24	10,5	11	0	3	4	9	-1.600
MD ₁₈	18	9	9	0	2	3	6	-1.861
MD ₁₉	25	2,5	6	3	1	5	11	-0.640

Figure 2. Helical wheels projections of MD₁₁ and its truncated analogs. The projections were realized using online program available at <http://rzlab.ucr.edu/scripts>. The hydrophilic residues are presented as circles, hydrophobic residues as diamonds, potentially negatively charged as triangles, and potentially positively charged as pentagons. The most hydrophobic residues are presented in green, and the amount of green is decreasing proportionally to the hydrophobicity (with zero hydrophobicity coded as yellow). Hydrophilic residues are coded red, and the amount of red is decreasing proportionally to the hydrophilicity. The charged residues are light blue. The peptide length, the amino acid composition, and the GRAVY index of each peptide are indicated in the Table 1. The parameters were calculated using EMBOSS program.

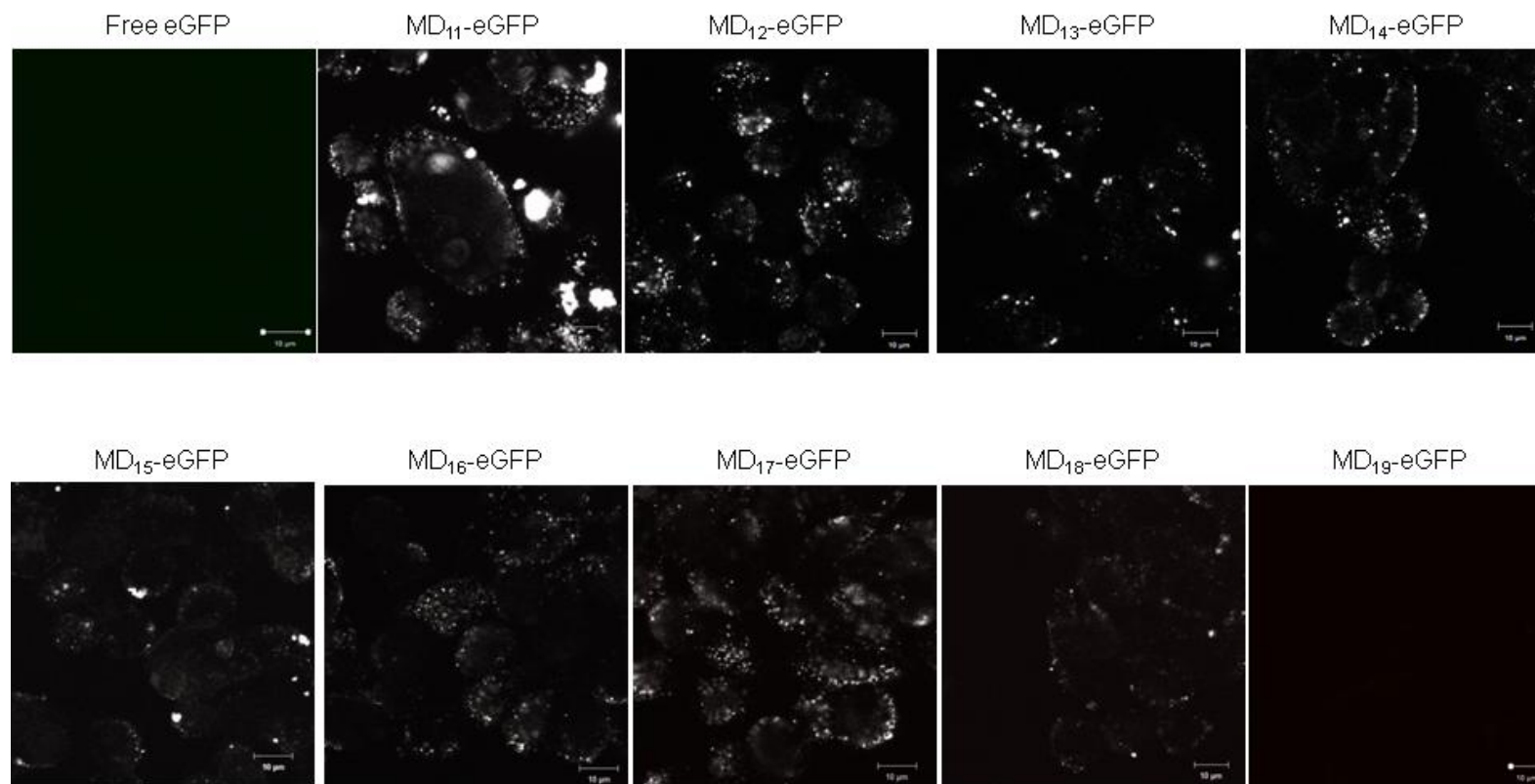


Figure 3. Confocal laser scanning microscopy images of living HeLa cells incubated for 4 hours with 0.3 μM of free eGFP and MD_x-eGFP fusion proteins. 16 successive optical slices were captured along the cellular z-axis with a step of 1 μm. The presented images correspond to the middle plan of cellular z-axis sectioning.

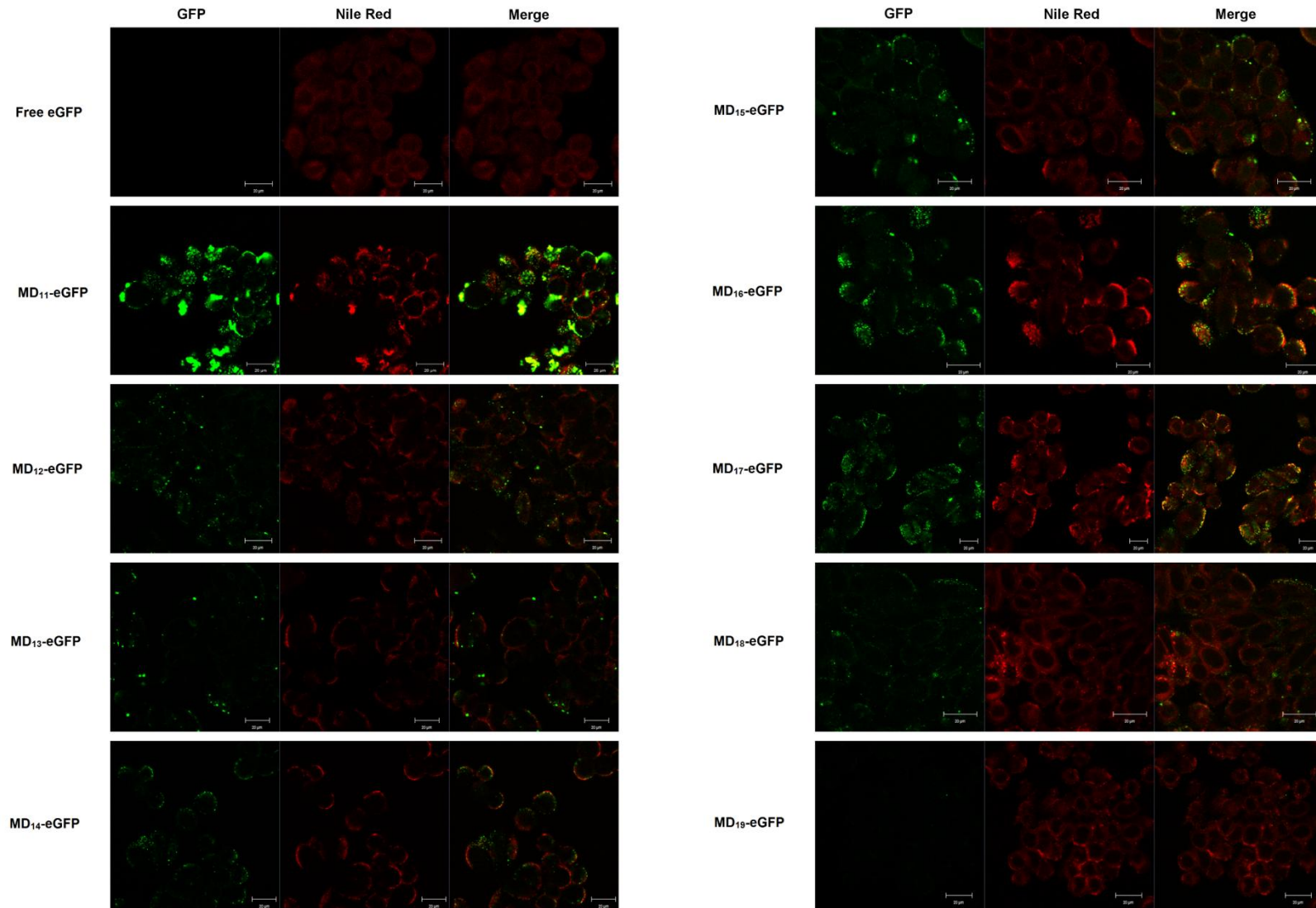


Figure 4. Intracellular localization of MD_x-eGFP in HeLa cells. Confocal microscopy images of fixed HeLa cells after a 4- hour incubation with 0.3 μ M of free eGFP and MD_x-eGFP fusion proteins. MD_x-eGFP signals are shown in green and the cellular lipid component in red. The showed images correspond to the central plan of cellular z-axis sectioning.

degree of intracellular fluorescence accumulation varied between the different MD_x constructions.

4.3. Cellular uptake assays

To quantify the cellular uptake of MD₁₁ and its truncated forms, FACS analysis was performed after treatment of living HeLa cells with 0.3 μM of each fusion protein after 4 hours of incubation. The extra-bound fluorescent proteins were removed by trypsin digestion. All peptides were found to penetrate into cells, but the new derived MD₁₁ peptides differed remarkably in efficiency of penetration (Fig. 5A). No improvement of uptake was observed when the number of MD₁₁ amino acid residues was further reduced. Indeed, the maximum efficiency of internalization was observed in HeLa cells incubated with MD₁₁-eGFP.

Endocytosis inhibitors were then used to investigate the molecular mechanism of penetration of MD₁₁ and its truncations in target cells. We analyzed three individual endocytic pathways. Nystatin was employed to inhibit the caveolae-mediated endocytosis, wortmannin to inhibit macropinocytosis, and MβCD to disrupt the import through lipid rafts. Pre-incubation of HeLa cells with these compounds had no inhibitory effect on the cellular uptake of MD₁₁-eGFP (Fig. 5B). In contrast, a decreased uptake of MD₁₂₋₁₉ truncations was observed after nystatin and wortmannin treatment, indicating that their uptake mainly proceeded via caveolae-mediated endocytosis and macropinocytosis. Thus, MD₁₁ is the shortest CPP derived from the EBV ZEBRA transcription factor with the highest direct transfer efficiency by an endocytosis-independent mechanism.

4.4. Confocal imaging of MD₁₁ peptide-lipid interaction

To further explore the interaction of MD₁₁ with lipids, we analyzed the MD₁₁-eGFP internalization in lipid vesicles. Interaction of MD₁₁ with pre-formed GUVs was monitored by confocal laser scanning microscopy. Nile Red dye staining was performed to visualize lipid vesicles. All the vesicles were homogeneously stained with the red dye. Images were recorded 1, 2.5, 3.5, 4.5 and 24 hours after MD₁₁-eGFP addition. We noticed a time-dependent accumulation of green fluorescent signal on membranes. The co-localization of the peptide with the lipids was revealed by the yellow color resulting from the overlay of Nile Red and eGFP signals. Three different patterns of interactions were observed. After 3.5h incubation, a

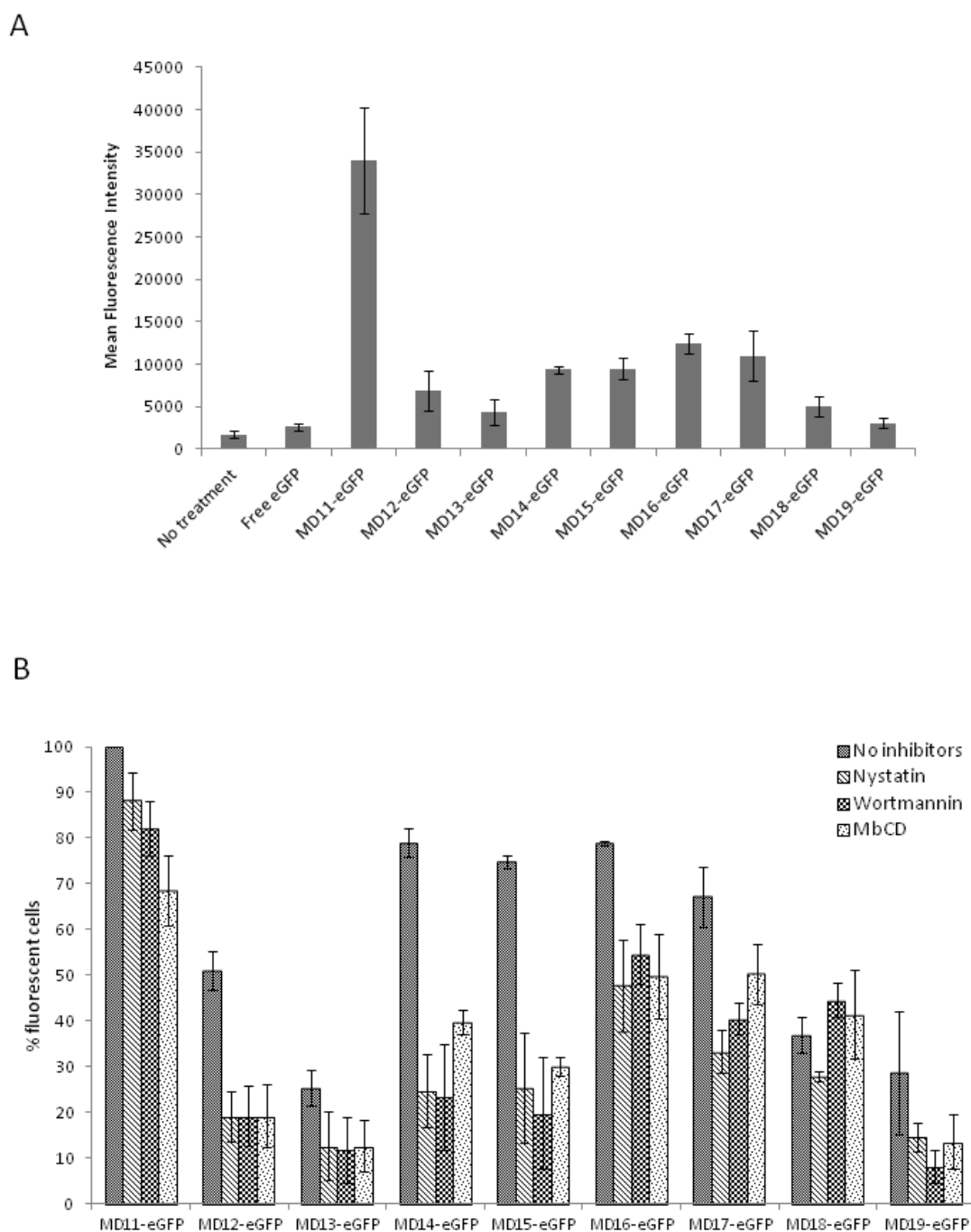


Figure 5. FACS cellular uptake of MD₁₁ and its truncated analogs. A) HeLa cells were incubated with 0.3 μ M of different peptides fused to eGFP for 4h at 37°C. The data are expressed as mean fluorescence intensity (MFI) of eGFP positive cells and the mean \pm s.d. of four independent experiments. B) Uptake of MD₁₁ and its truncated analogs in presence of endocytosis inhibitors. HeLa cells were pre-treated with 50 μ g/mL nystatin, 0.1 μ M wortmannin or 5mM M β CD for 30min at 37°C. 0.3 μ M of recombinant proteins were incubated with cells for 4h at 37°C. The data are expressed as % of eGFP positive cells and the values indicated are the mean \pm s.d. of two independent experiments. Fluorescence values have been normalized assuming as 100% the number of fluorescent cells incubated with MD₁₁-eGFP without inhibitors.

strong co-localization of MD₁₁ peptide was recorded on semi-fluid and rigid vesicles containing both SM and cholesterol (Fig. 6B and 6C). No interaction was observed between MD₁₁ and PC fluid vesicle membranes even after 24 hours (Fig. 6A). Curiously, a topical accumulation of MD₁₁ was observed on semi-fluid vesicles (starting after 1 hour incubation).

5. Discussion

Increasing efforts are currently being made for developing CPPs as delivery systems for therapeutic macromolecules. In this context, the identification of their cellular uptake mechanisms is essential for optimization of appropriate strategies. The majority of CPPs is internalized via endocytosis when coupled to cargo molecules [13-15]. This aspect represents a great limit because payload molecules can be entrapped and degraded into endosomal vesicles, with the consequent loss of their biological activity. Therefore, identification of new CPPs endowed with direct translocation mechanism is necessary. It has been shown that EBV ZEBRA protein contains a 51 amino acid- sequence (residues 170-220, MD) able to transduce across mammalian cellular membranes by an endocytosis-independent mechanism [16]. MD peptide contains two regions: the basic DBD and the adjacent leucin-rich DIM domain. Previous results indicated that the presence of both domains could be essential for MD cellular uptake [16]. Furthermore, enzymatic activity has been detected even in cells transduced with MD fused to high-molecular weight protein, such as β -galactosidase (116kDa) [16]. This evidence prompts us towards further investigations to explore the employment of MD as carrier of therapeutic macromolecules. For this purpose, different MD truncations have been designed to reduce the carrier size maintaining its ability to pass through cell membranes by an endocytosis-independent mechanism. As the presence of a cargo may influence the internalization process, MD peptides were fused to the eGFP reporter protein and their cellular uptake was investigated by confocal microscopy and FACS. Starting from an eight amino acid shorter peptide (MD₁₁), six different peptides were produced by reducing its C-terminal end, and two were generated by isolation of DBD (MD₁₈) and the DIM (MD₁₉) domains. We observe that all DIM-deleted peptides possess protein transduction properties, even though the highest efficiency has been observed in cells incubated with MD₁₁-eGFP. Unexpectedly, we find that the DBD domain alone (MD₁₈) maintains the internalization ability, whereas peptide containing only the DIM domain (MD₁₉) is not able to enter cells. Thus, the internalization is clearly associated with the cationic DBD and not with

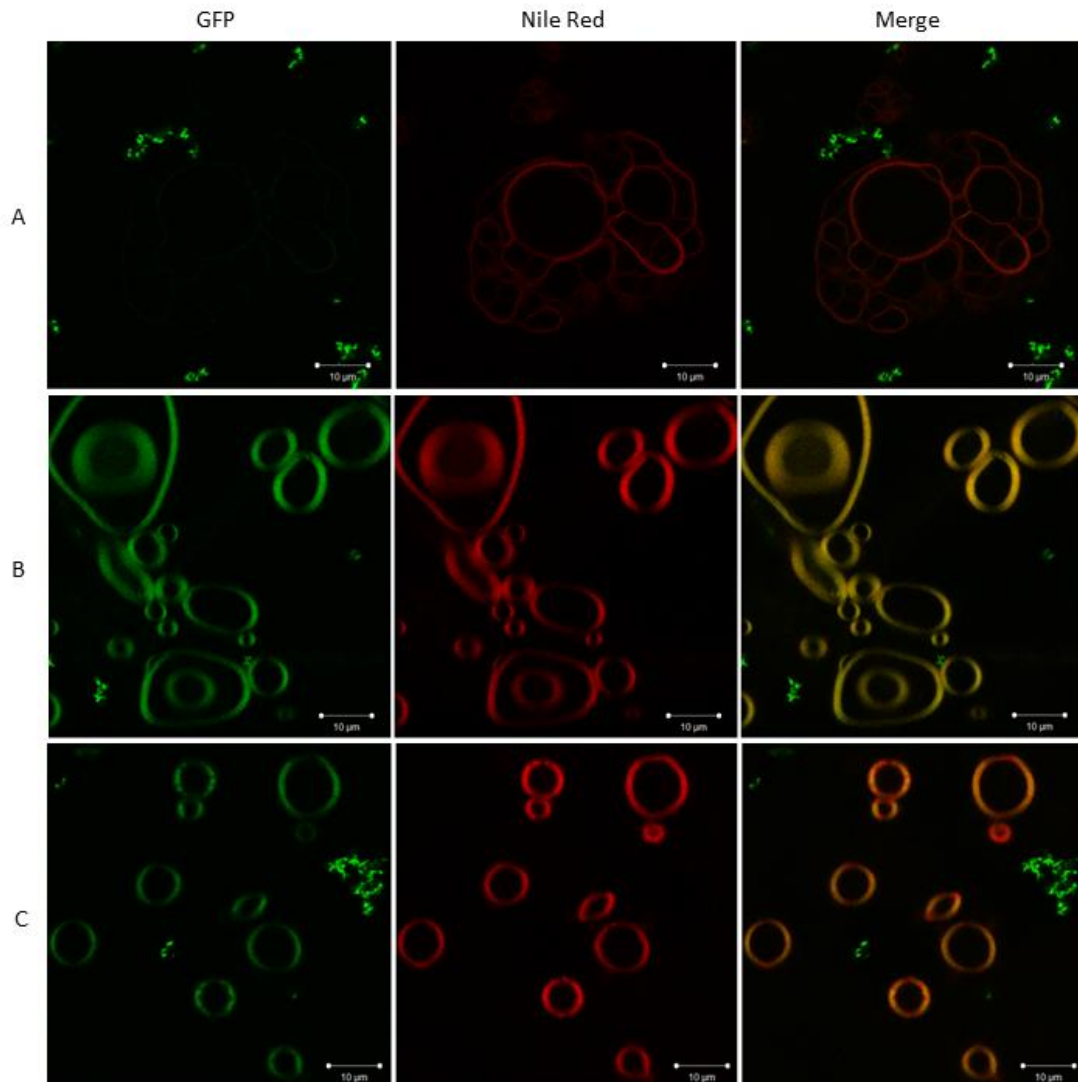


Figure 6. Confocal imaging of MD₁₁eGFP-GUV interaction after 3.5h treatment. MD₁₁-eGFP was incubated with A) fluid (100% PC), B) rigid (SM:Chol 50:50 % molar ratio) and C) semi-fluid (PC:SM:Chol 30:30:30 % molar ratio) GUVs. MD₁₁-eGFP signal is shown in green and the lipids in red. The membrane co-localization of the peptide is indicated by the yellow colour.

the DIM domain. To quantify the peptide cellular uptake, FACS analysis was performed. In agreement with qualitative data obtained by confocal microscopy, progressive shortening of the DIM domain correlates to a decrease in translocation efficiency. MFI values normalized to the highest recorded signal of MD₁₁ show a decrease of about 70% for all DIM-deleted peptides and the absence of internalization for MD₁₉-eGFP (DIM domain only).

We can first explain these results considering that the decrease in length of DIM domain could entail structural modifications of MD₁₁ helix affecting its penetrating activity. Secondly, as shown for Tat peptide [21], MD₁₁ could penetrate into cells in dimer form and the reduction of the DIM sequence might prevent this process inducing a decrease in the internalization efficiency.

Curiously, the loss in uptake is not directly proportional to the number of removed amino acids. In fact, we observe that the deletion of five or seven residues (MD₁₂ and MD₁₃ respectively) induces a lower cytoplasmic accumulation than the uptake mediated by MD₁₆ and MD₁₇. The analysis of the primary sequences shows that, similarly to MD₁₁, both MD₁₆ and MD₁₇ possess a repeated motif at C-terminal end. This motif is composed of two or three successive hydrophobic residues (Leu or Ala) followed by a basic (Lys) and/or a polar (Gln or Ser) residue. Probably, the presence of this motif is involved in the interaction with hydrophobic membrane lipids and may regulate the cellular uptake. These pieces of evidence highlight that the length of the MD helix and its polar character play a key role in the internalization process.

To check the impact of chain length reduction on internalization mechanism, we investigated the uptake of MD₁₁ and its derived peptides in presence of endocytosis inhibitors. Inhibition of caveolae-mediated endocytosis and macropinocytosis does not affect MD₁₁-eGFP uptake, whereas the internalization of all MD₁₁ deleted forms is strongly impaired. This loss of endocytosis-independent mechanism is also suggested by the presence of vesicles-like structures in cytoplasm of HeLa target cells incubated with MD₁₆ and MD₁₇ fusion proteins (Fig. 4). These results demonstrate that (i) the MD₁₁-mediated cellular import is neither based on caveolae-mediated endocytosis nor on macropinocytosis and (ii) the deletion of DIM domain induces a switch from a non-endocytic to an endocytic pathway. Future optimizations

of MD₁₁-derived CPPs need to take into account that all the residues of the DIM domain are required to guarantee the endocytosis-independent internalization.

To elucidate the roles of lipids in MD₁₁-membrane interactions, we used fluid (PC), rigid (SM, Chol) and semi-fluid (PC, SM, Chol) vesicles. We show that MD₁₁ peptide strongly interacts with sphingomyelin and cholesterol, but not with phosphatidylcholine. Microscopy images well correlate with FACS data confirming that depletion of cholesterol from HeLa cell membranes by treatment with M β CD lead to a low decrease of MD₁₁ entry. Since SM and cholesterol are both involved in lipid raft formation [22], we suggest that MD₁₁ may interact with lipid-raft membrane domains during internalization. However, any significant accumulation in vesicles' lumen has been detected after MD₁₁-eGFP incubation. Our findings are in agreement with results on other CPPs such as R12-HA, Pep-1 and MPG, known to enter cells in endocytic-independent manner, were not able to translocate and accumulate in artificial vesicles [23-25]. These studies have established that direct translocation is strongly dependent on the presence of a negative membrane potential, and is modulated by the lipid composition, the membrane curvature and the local peptide concentration [23, 24, 26]. In any case, artificial membranes represent a simplified system compared to biological membrane, and a combination of different methods is necessary to fully clarify the MD₁₁ entry mechanism.

Recent studies have reinforced the notion that a requirement for direct translocation of CPPs is the presence of a hydrophobic moiety and have proved that this process occurs at specific locations of the plasma membrane [24, 27]. The attachment of a hydrophobic peptide tag deriving from human influenza hemagglutinin greatly accelerates the direct penetration of dodeca-arginine (R12) cationic peptide [24]. The hydrophobicity of the coupled peptide stimulates dynamic morphological alterations in the plasma membrane, which allow the permeation through the lipid bilayer [24]. Since the DIM domain of the MD₁₁ is hydrophobic, we believe that it acts in a similar way and can stimulate the internalization via direct penetration. To support this hypothesis, the MD crystal structure describes two helices (DBD and DIM) linked to a loop that confers flexibility to peptide [19]. According to these pieces of evidence, we can claim that the charged DBD domain is involved in the initial interaction with the bilayer while the hydrophobic domain is crucial for insertion.

6. Conclusion

The goal of this study was to define the role of the amino acid composition of the ZEBRA MD in the uptake process. We have demonstrated that the MD₁₁ C-terminal DIM domain contributes to the internalization but doesn't act as a driving force in this process. Our results indicate that the DIM domain regulates the MD₁₁ endosomal escape. This amino acid sequence can be used for engineering novel endosomal escaping CPPs. Furthermore, MD₁₁ can be used as a carrier for delivering of biologically active cargo molecules with therapeutic potential.

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CHAPTER II

MD₁₁-mediated delivery of the translational factor eIF3f

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Preface part I

The regulation of the protein synthesis has a crucial role in governing the eukaryotic cell growth. Minimal defects in the translational machinery can alter the cellular physiology and lead to cell malignancy or cell death. Among the different factors intervening in the regulation of this process, a contribution to oncogenesis and maintenance of the cancer state has been reported for the eukaryotic initiation factor 3 (eIF3) (Goh *et al.*, 2011; Zhang *et al.*, 2007; Zhang *et al.*, 2008). This complex is composed of 13 subunits (designated eIF3 a-m). The expression of eIF3 subunits is altered in several cancers, and in particular the f subunit (eIF3f) is significantly downregulated in pancreas, vulva, breast, melanoma, ovary and small intestine tumors (Shi *et al.*, 2006).

The first part of this chapter contains a review on the structure and function of the eIF3 complex and, in particular, the function of the eIF3f subunit. This study highlights the seemingly paradoxical roles that eIF3f plays in the regulation of protein synthesis in proliferating/cancer cells and in post-mitotic differentiated muscle cells. Indeed, the eIF3f subunit acts as ‘negative repressor’ of translation initiation (Shi *et al.*, 2003) and ‘death executor’ in cancer cells (Shi *et al.*, 2006), and as ‘translational and cell growth enhancer’ in muscle cells (Lagirand-Cantaloube *et al.*, 2008).

The aim of this bibliography study was to delineate the global eIF3f interactome and clarify its intracellular role both in cancer and in muscle cells. Since the conclusions drawn by the various studies on eIF3f role in different cell types point in opposite directions (hence the definition ‘ambivalent’), a complete understanding in terms of how eIF3f affects protein synthesis and cell metabolism still remains to be reached. The eIF3f cell-specific action could be determined either by the interaction with molecular partners differently expressed in malignant/normal cells or by its ability in discriminating specific pools of mRNAs (*i. e.* mRNAs encoding for proteins involved in cell proliferation) and their subsequent translation according to the cell cycle phase and the cell type.

Given the central role in different intracellular pathways and in the balance between cell proliferation and apoptosis, eIF3f represents a valid candidate for biotherapeutic applications.

The translational factor eIF3f: the ambivalent eIF3 subunit

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1. Abstract

The regulation of the protein synthesis has a crucial role in governing the eukaryotic cell growth. Subtle changes of proteins involved in the translation process may alter the rate of the protein synthesis and modify the cell fate by shifting the balance from normal status into tumoral or apoptotic one. The largest eukaryotic initiation factor involved in translation regulation is eIF3. Amongst the 13 factors constituting eIF3, the f subunit finely regulates this balance in a cell-type specific manner. Loss of this factor causes malignancy in several cells, and atrophy in normal muscle cells. The intracellular interacting partners which influence its

physiological significance in both cancer and muscle cells are detailed in this review. By delineating the global interaction network of this factor and by clarifying its intracellular role, it becomes apparent that the f subunit represents a promising candidate molecule to use for biotherapeutic applications.

2. Introduction

Protein synthesis is one of the most complex and energy-consuming processes in eukaryotic cells, by which the genetic information is translated from a nucleic acid code into an amino acidic language to make proteins. Global protein synthesis rates have a key role in governing cell growth, the control of cell size, and cell proliferation. Changes in the levels of specific regulatory proteins involved in the translation process may affect the translation rates and the amount of produced proteins. Thus, the regulation of gene expression is a crucial step in cellular physiology considering that subtle defects in the mechanism of translational control may lead either to cell malignancy or cell death. Protein synthesis is mainly regulated at the initiation step. In eukaryotes, initiation can be divided into two steps: formation of a 48S initiation complex and its joining with a 60S subunit [1]. First, the eukaryotic initiation factors eIF3, eIF1, eIF1A and eIF2-GTP-Met-tRNA_i bind to the 40S ribosomal subunit to form a 43S preinitiation complex. This complex subsequently attaches to the 5'-proximal region of mRNAs, after which, eIF4A, -4B and -4F unwind their secondary structure. The 43S complex then scans the 5' untranslated region in the 5' to 3' direction until the initiation codon, where it stops and forms a 48S complex with codon-anticodon base-pairing through eIF5. Second, eIF5B promotes the displacement of the eukaryotic initiation factors and the subsequent joining of 48S complex with 60S subunit [1].

The largest and more complex eukaryotic initiation factor, which plays a role in translation regulation, cell growth and cancer, is eIF3. This review is focused on the function of the f-subunit of eIF3 (eIF3f) in the translation regulation process. An analysis of its physiological significance and its intracellular interacting partners involved in translational regulation both in cancer and muscle cells, is sequentially detailed. The aim is to delineate the global eIF3f interaction network and clarify its intracellular role in order to validate eIF3f as a lead candidate to use for biotherapeutic applications.

3. Eukaryotic Initiation Factor 3

Translation initiation in eukaryotes is an intricate process requiring at least nine eukaryotic initiation factors [1,2]. Among these factors, eIF3 is the largest as it comprises up to 13 non-identical subunits. Several functions have been ascribed to eIF3, including the interaction and stabilization of eIF2-GTP-Met-tRNA_i ternary complex, binding to the 40S ribosomal subunit, facilitating the binding of mRNA to the 40S ribosome, and promoting dissociation of 40S and 60S ribosomal subunits. Other initiation factors (*e. g.* eIF4G, eIF4B, eIF5 and eIF1) are known to interact with eIF3, suggesting that this factor might have a role in ‘organizing’ proteins on the surface of the 40S ribosome [3-5].

3.1. Structure and nomenclature

Mammalian eIF3 is an 800kDa molecular mass assembly of 13 subunits that are designated eIF3a to eIF3m [3-11]. In the past, ad hoc nomenclatures for eIF3 subunits have been derived from subunit molecular weights or gene names. In 2001, to eliminate confusion surrounding cross-species comparisons of eIF3 subunits, a unified nomenclature was proposed by Browning *et al.* [7]. Each subunit is identified by a letter designation based on the decreasing order of the mammalian subunit masses determined from SDS-PAGE mobility (Table 1). For two novel subunits, -l and -m, the nomenclature has been adapted as they have arisen.

Initial characterizations of mammalian eIF3 were based on biochemical methods applied to purified proteins from rabbit reticulocytes and HeLa cells [12, 13]. Due to its large size and complexity, a detailed understanding of eIF3 structure and function has been achieved more recently from experiments based on the cloning and characterization of human cDNAs encoding the eIF3 subunits [5, 14-16]. By comparing the cDNA sequences of the mammalian eIF3 subunits to the entire genome of the budding yeast *Saccharomyces cerevisiae*, it appears that only five mammalian subunits eIF3-a, -b, -c, -g and -i have identifiable homologues encoded in yeast (Table1). A sixth ortholog, eIF3j, is a nonessential subunit of the budding yeast eIF3 that enhances interactions with other eIFs [17], promotes binding of eIF3 to the 40S subunit [18] and has an independent function in 40S ribosome biogenesis [19]. Biochemical interactions have been detected between these five conserved

Table 1. Summary of eukaryotic eIF3 subunits. The ‘conserved’ core subunits are highlighted in bold type, the ‘functional’ core subunits are indicated in blue and the ‘dispensable’ ones in grey.

Unified nomenclature	<i>S. cerevisiae</i> + MW	<i>H. sapiens</i>	<i>S. pombe</i>	<i>A. thaliana</i>	Consensus motif
eIF3a	Tif32 - 110 kDa	p170	p107	p114	PCI
eIF3b	Prt1 - 90 kDa	p116	p84	p82	RRM
eIF3c	Nip1 - 93 kDa	p110	p104	p105	PCI
eIF3d	-	p66	Moe1 ^a	p66	-
eIF3e	-	p48	Int6 ^a	p51	PCI
eIF3f	-	p47	Csn6	p32	MPN
eIF3g	Tif35 - 33 kDa	p44	Tif35	p33	RBD
eIF3h	-	p40	p40 ^b	p38	MPN
eIF3i	Tif34 - 39 kDa	p36	Sum1	p36	WD repeats
eIF3j	Hcr1 - 35 kDa	p35	-	-	-
eIF3k	-	p28	-	p25	PCI
eIF3l	-	p67	-	p60	PCI
eIF3m	-	GA17	Csn7B ^b	-	PCI

^a Subunits contained in *S. pombe* Int6 eIF3 complex

^b Subunits contained in *S. pombe* Csn7B eIF3 complex

Abbreviations: MPN Mpr1p and Pad1p N-terminal conserved domain; PCI 26S proteasome, COP9 signalosome and eukaryotic initiation factor eIF3 conserved domain; RBD RNA-binding domain; RRM RNA-recognition motif; S6K1 ribosomal protein S6 kinase 1; WD conserved regions of approximately 40 amino acids typically bracketed by Trp-Asp.

subunits in both mammalian and yeast system [14,15, 20]. The *S. cerevisiae* eIF3 complex also contains 3 additional proteins, p135, p62 and p16, for which corresponding homologs are not found in the mammalian complex [21-23], whereas 7 subunits (eIF3-d, -e, -f, -h, -k, -l and -m) identified in mammalian eIF3 complex are absent in the yeast complex (Table1). Most of these 7 mammalian subunits of eIF3 appear to be highly conserved in *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana* and the fission yeast *Schizosaccharomyces pombe* [4, 5]. Nevertheless, the biochemical function of this complex is conserved inasmuch as eIF3 purified from *S. cerevisiae* can replace mammalian eIF3 in the *in vitro* methionyl-puromycin synthesis assay [21]. Accordingly, mammalian eIF3 complex consists of five subunits (eIF3-a, -b, -c, -g, -i) that represent a ‘conserved’ core essential for translation initiation *in vivo* and the remaining ‘noncore’ subunits that appear to be evolutionarily divergent and serve to modulate its activity [5, 14, 20].

The function of each subunit of eIF3 has been investigated in *S. cerevisiae* first. Deletion or mutation of eIF3a [24, 25], eIF3b [15, 26], eIF3c [27], eIF3g [28], or eIF3i [20, 28, 29] in yeast leads to a vast reduction in protein synthesis, suggesting that each of these five subunits is required for eIF3 integrity. The role of each subunit and the minimum combination of components required for the formation of an active and functional core have been investigated in mammalian eIF3 also [30]. By using a baculovirus-based coexpression system, recombinant eIF3 complexes lacking different individual subunits have been reconstituted. Remarkably, deletion studies showed that the rate of the protein synthesis does not change by knockdown the two evolutionarily conserved subunits, eIF3g and eIF3i in HeLa cells, and the eIF3 activity remains unaltered after deletion of the three non-conserved subunits eIF3-d, -k, -l [30]. Therefore, eIF3-g, -i, -d, -k, -l subunits are not essential for eIF3 activity but might be involved in translational control of specific mRNAs or in particular cellular conditions [30]. Hence, the functional core of mammalian eIF3 consists of three conserved subunits eIF3-a, -b, -c, and three non-conserved subunits eIF3-e, -f and -h. These three last subunits might serve to stabilize the eIF3-abc complex which directly participates in translation initiation [30]. *In vitro* stepwise reconstitution of human eIF3 subunits has revealed the architectural features of this factor. The dimer of subunits eIF3a and eIF3c serves as a central scaffold to which most of the other subunits bind. This dimer directly interacts with the subunits eIF3e, -k, -l and -m containing the PCI domain (the 26S proteasome, COP9 signalosome and eukaryotic initiation factor eIF3 conserved domain) and with the subunits

eIF3-f and-h containing a MPN (Mpr1p and Pad1p N-terminal conserved domain) to form a highly stable PCI/MPN octamer. Subunits -d, -b, -g, -i, and -j then finalize the assembly of the eIF3 complex [11].

3.2. eIF3 function in cell cycle regulation, translational regulation, and cancer

The global protein synthesis in mammals occurs mainly in the G1 phase of a cell cycle and is reduced during mitosis. This process is modulated by several translation initiation factors [31]. The first studies that associated two eIF3 subunits, eIF3a and eIF3b, to cell cycle regulation have been performed in *S. cerevisiae*. Both subunits are essential for the G1-S phase transition in yeast [32,33]. A study on the eIF3a expression in mammalian cells has shown that its levels oscillate during cell cycle and its maximum of expression is detected in S phase, indicating that eIF3a may be a translational regulator for proteins important for S phase entrance [34]. Additionally, the subunits eIF3a [35], eIF3e [36, 37], eIF3k [38] and eIF3f [39] have been found in both cytoplasm and nucleus, indicating that these factors may shuttle between the two subcellular localizations according to cell cycle progression.

Proper execution of the cell cycle requires the synthesis and activation of key proteins at specific times. The most prevalent mechanism for regulating the overall rate of protein synthesis involves the phosphorylation of the initiation factors. Besides phosphorylation, the rate of protein synthesis is regulated by other mechanisms and post-translational modifications of the translational machinery; such as methylation of lysine or arginine residues and O-glycosylation [40]. Recent reports confirm the role of the eIF3 factor in regulation of translation initiation rate [40-42]. However, the molecular mechanisms by which the function of eIF3 is regulated, are poorly understood. A mass spectrometric approach has been used to determine post-translational modifications that regulate the activity of eIF3 during translation initiation process (Table 2). A total of 29 phosphorylation sites and several other post-translational modifications (loss of N-terminal methionine and/or N-terminal protein acetylation) have been recently identified [10].

Alternatively, an indirect regulation of the activity of eIF3 is performed by association of its subunits with other proteins involved in the regulation of protein synthesis. For example, the subunit eIF3e binds p56 in interferon-treated or virus-infected mammalian cells,

Table 2. Identification of eIF3 protein subunits and their corresponding post-translational modifications using a mass spectrometric analysis [10].

Protein name	UniProt accession number	Molecular mass ^a	Sequence coverage	Post-translational modifications ^b
		Da	%	
eIF3a	Q14152	166,758.3	86	Loss of Met-1, phosphorylation (Ser-881, Ser-1198, Ser-1336*, Ser-1364*)
eIF3b	P55884	93,093.7	77	Acetylation (Met-1), phosphorylation (Ser-83, Ser-85, Ser-119, Ser-125*, Ser-152, Ser-154, Ser-164)
eIF3c	Q99613	106,143.8	65	Phosphorylation (Ser-9, Ser-11, Ser-13, Ser-15, Ser-16, Ser-18, Ser-39, Ser-166**, Thr-524*, Ser-909**)
eIF3d	O15371	63,972.9	74	Not found
eIF3e	P60228	52,131.8	84	Loss of Met-1, acetylation (Ala-2)
eIF3f	O00303	37,554.8	79	Loss of Met-1, acetylation (Ala-2), phosphorylation (Ser-258*)
eIF3g	O75821	35,639.8	83	Loss of Met-1, phosphorylation (Thr-41, Ser-42)
eIF3h	O15372	40,010.4	89	Phosphorylation (Ser-183*)
eIF3i	Q13347	36,501.9	93	Not found
eIF3j	O75822	29,293.2	81	Loss of Met-1, acetylation (Ala-2), phosphorylation (Ser-11, Ser-13, Ser-20, Thr-109**)
eIF3k	Q9UBQ5	24,970.6	75	Loss of Met-1, acetylation (Ala-2)
eIF3l	Q9Y262	66,637.9	70	Loss of Met-1, acetylation (Ser-2)
eIF3m	Q7L2H7	42,413.8	74	Loss of Met-1, acetylation (Ser-2)

^a Calculated from the theoretical average mass of the corresponding eIF3 protein subunit plus any post-translational modifications identified.

^b *, found only after TiO₂ phosphopeptide enrichment **, found only after Ga(III) IMAC or TiO₂ phosphopeptide enrichment.

Table 3. Translational factor alterations in cancers.

Subunit	Observed modification	Cancer association
eIF3a	Increased expression	Mouse melanoma, human breast, cervical, esophageal, lung, and gastric cancers
eIF3b	Increased expression	Human breast carcinoma
eIF3c	Increased expression	Human testicular seminomas
eIF3e	Decreased expression	Human breast and lung carcinomas
eIF3f	Decreased expression	Pancreas, vulva, ovary, breast, small intestine tumors, and melanoma
eIF3h	Increased expression	Human breast, prostate, hepatocellular carcinomas
eIF3i	Increased expression	Cadmium transformed NIH3T3 cell lines
eIF3m	Increased expression	Human colon cancer

and inhibits the translation *in vitro* and *in vivo* [43, 44]. The subunit eIF3g interacts with Paip1, a Poly (A)-binding protein and stimulates translation initiation [45] whereas the subunits eIF3h and eIF3f interact with TRC8, a ubiquitin E3 ligase, and inhibit protein synthesis, possibly through ubiquitilation of eIF3 or some other translational components [46]. These mechanisms and interacting partners render eIF3 a pivotal player in controlling the protein synthesis and degradation.

A proper level of translation initiation is necessary to regulate cell proliferation since the hyperactivation or the down-regulation of the rate of the protein synthesis contributes importantly to cell malignancy. The contribution of eIF3 to oncogenesis and maintenance of the cancer state has been demonstrated in several studies [47-49]. Amongst the 13 subunits constituting eIF3, overexpression of subunits eIF3-a, -b, -c, -h, -i, and -m has been detected in several different solid tumors and in several different cancer cell lines (Table 3, for literature citations, see [42, 47, 48]). The correlation of abnormal eIF3 subunit levels and cancer, indicates that eIF3 has an important role in determining the balance between cell proliferation and apoptosis. A study performed on NIH3T3 fibroblasts stably transfected with five individual eIF3 subunit cDNAs, has confirmed that their overexpression contributes to malignant phenotype [48]. Ectopic overexpression of eIF3-a, -b, -c, -h or -i induces an increase in protein synthesis rate, the resistance to apoptosis and the oncogenic transformation of NIH3T3 cells. Hence, eIF3 may play a causal role in neoplasia and the misregulation of translation may be a contributory factor to cancer pathology [48]. It has been demonstrated that the overexpression of a single eIF3 subunit causes malignant transformation and induces an increase in eIF3 activity, which in turn stimulates the translation of mRNAs involved in cell proliferation. Zhang *et al.* have suggested that transforming subunit overexpression leads to the preferential stimulation of so-called “weak” mRNAs, which are translated more efficiently considering the abundant presence of secondary structures in their 5'-UTRs. Generally, “weak” mRNAs encode proteins involved in promoting cell growth and proliferation, such as c-myc, cyclin D1 and growth factors. Thus, the increase of “weak” mRNA products stimulates the malignant transformation of cells [48].

Two other eIF3 subunits, eIF3e and eIF3f, are downregulated in human breast/lung carcinomas and melanoma/pancreatic cancer respectively [50-54]. Low eIF3e expression is considered as a significant factor in predicting poor prognosis for nonsmall cell lung cancer

[52]. It may be likely that these factors have a causal role in cancer development inasmuch as their decrease removes the restraints that block the protein synthesis and stimulates the selective translation of oncogenic proteins involved in cell proliferation [53, 54].

4. The f subunit of eIF3

In 2003 Shi *et al.* have shown, for the first time, that in human cells the noncore f subunit of the mammalian eIF3 multiprotein complex acts as a negative regulator of translation [39]. Furthermore, in mammalian cells eIF3f inhibits protein synthesis at the translational level and not by affecting mRNA levels [55]. Translation initiation can be cap-dependent or cap-independent/IRES-dependent. It is not clearly known whether eIF3f inhibits specifically one of these two types of translation. However, the silencing of eIF3f increased both cap-dependent and IRES-dependent translation, indicating a suppressive role of eIF3f on both translation initiation mechanisms [56]. Furthermore, it has been recently demonstrated that the f subunit plays a role in the inhibition of HIV-1 replication. Overexpression of N-terminal 91 amino acids of eIF3f or full-length eIF3f drastically restricts HIV-1 replication by interfering with the 3'-end processing of HIV-1 mRNAs and reducing their nuclear and cytoplasmic levels [57, 58].

4.1. Intracellular localization

Under normal conditions most of the endogenous eIF3f is bound to 40S ribosomal subunit [55]. However, confocal microscopy images of human melanoma cells show that eIF3f is localized both at cytoplasmic and at nuclear level [39]. Other eIF3 subunits, such as eIF3a, eIF3e and eIF3k, have been reported to have nuclear localization [35-38]. In fact, there are at least two different types of eIF3 complexes localized both in the cytoplasm and in the nucleus of cells. Hence, eIF3 is considered as a 'dynamic' complex, where 'dynamic' means that it may contain different and a variable number of subunits [59, 60]. The cytoplasmic eIF3 complex contains the subunits eIF3a-c and f, whereas the nuclear one lacks the subunits eIF3a and f [60]. What correlates this finding and the microscopy results?

It has been demonstrated that the phosphorylation of eIF3f may specifically increase its association with other eIF3 core complex subunits. In particular, during apoptosis, eIF3f is

directly phosphorylated by the cyclin-dependent kinase CDK11p46 in living cells [60] and is incorporated into a micro-complex containing eIF3b and eIF3c, but not eIF3a. By cell fractionation, it has been observed that this complex precipitates with the nucleus in the insoluble fraction of the cells. Nevertheless, eIF3f can also associate in a smaller complex consisting of eIF3a, eIF3b and eIF3c in the soluble fraction of both normal and apoptotic cells [60]. Therefore, under normal conditions the nuclear eIF3 contains eIF3b and eIF3c and, during apoptosis the phosphorylated eIF3f joins the nuclear complex. The model currently suggested is that the translation initiation can be regulated during apoptosis by the phosphorylated eIF3f binding to different sub-fractions of the eIF3 complex [60]. This finding, coupled with the fact that eIF3f is a Mov34 family member, led Shi *et al.* to elaborate the hypothesis that this factor may have a nuclear function in mRNA metabolism such as splicing or transport [39]. It is also possible that the nuclear eIF3 complex has functions other than translation initiation. For example, the synthesis of ribosomes in eukaryotes takes place in nucleus and then ribosomes are exported to the cytoplasm. Shi *et al.* suggest that CDK11p46 may regulate the function of the nuclear eIF3 complex by phosphorylating eIF3f considering that cyclin-dependent kinases are involved in ribosome biogenesis and nucleolar organization [60].

4.2. Regulation of eIF3f activity

The eIF3f subunit is a Mov34 family member, containing an MPN motif, which is found in two other macromolecular complexes, homologous to eIF3, the COP9 signalosome and the 19S proteasome [5, 6, 61, 62]. The presence of a MPN domain in complexes with apparently divergent functions, such as protein synthesis, signal transduction and protein degradation, has been explained by the fact that this domain is probably necessary to promote complex assembly and is required for proper interactions between subunits of these complexes [63]. Recent crystallographic studies have revealed that there are two classes of MPN domains, one possessing a metalloprotease activity and another possessing a structural function [64, 65]. The subunits eIF3f and eIF3h contain the second type of MPN domain, which is responsible for protein-protein interactions and for protein stability [64, 65]. The crucial role of this domain in determining the cellular function and the intracellular behaviour of eIF3f has been confirmed by several studies. In 2006, Shi *et al.* have shown that this domain, and more precisely the amino acid region spanning residues 170-248, is responsible for the translation

inhibition function of eIF3f [55]. Furthermore, it has been demonstrated that an eIF3f activating phosphorylation site is located in the Mov34 domain [60]. The phosphorylation status of this subunit can significantly influence its association with eIF3 core complex, its intracellular localization, its function in the regulation of protein synthesis rate and in the control of the subtle balance between cell-growth and cell-death (see below). Moreover, several intracellular interacting partners of eIF3f have been identified (Figure 1) using yeast two-hybrid screens. In most identified partners the interactions are mediated by the Mov domain, suggesting that it may play a central role in regulating the activity of this subunit.

4.3. Apoptosis versus cell growth: the importance of eIF3f rate

4.3.1. Phosphorylation of eIF3f during apoptosis

Using a yeast two-hybrid system, Shi *et al.* have shown that endogenous cyclin-dependent kinase 11 (CDK11) and eIF3f protein can interact and their interaction can be strengthened by the stimulation of apoptosis [39]. The CDK11 kinases belong to a large family of p34cdc2-related kinases [66] containing at least 20 CDK11 isoforms that are differentially expressed in mammalian tissues and regulate diverse cellular functions. The largest CDK11p110 isoform is associated with cyclin L and various splicing factors, and is involved in the regulation of transcription and RNA splicing in proliferating cells [67, 68]. Expression of the CDK11p110 is ubiquitous and constant throughout the cell cycle [69]. During Fas- and tumor necrosis factor- induced cell death, CDK11p110 is activated by proteolytic cleavage by caspase-3. Caspase-3 processing removes the N-terminal part of the protein, which contains nuclear translocation signals and is responsible for protein stability, and generates a smaller 46-50kDa protein (CDK11p46) containing the catalytic kinase domain [70, 71].

After anti-Fas or staurosporine treatments in human melanoma cells, the caspase-processed C-terminal fragment CDK11p46 strongly interacts with eIF3f via its Mov34 domain [39] and, due to its kinase activity, phosphorylates eIF3f inducing the inhibition of translation (Figure 2). The mutation of the phosphate transfer site in CDK11p46 abrogates the phosphorylation of eIF3f protein. Two different phosphorylation sites have been identified in eIF3f during apoptosis: Ser46 [39] and Thr119 [60]. CDK11p46 phosphorylates both sites.

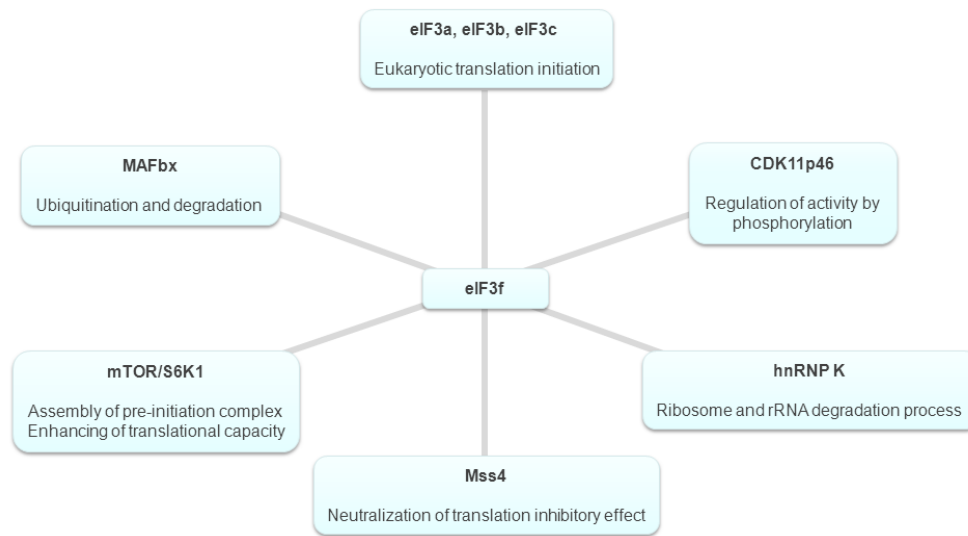


Figure 1. Schematic representation of the intracellular interacting partners of the eIF3f subunit.

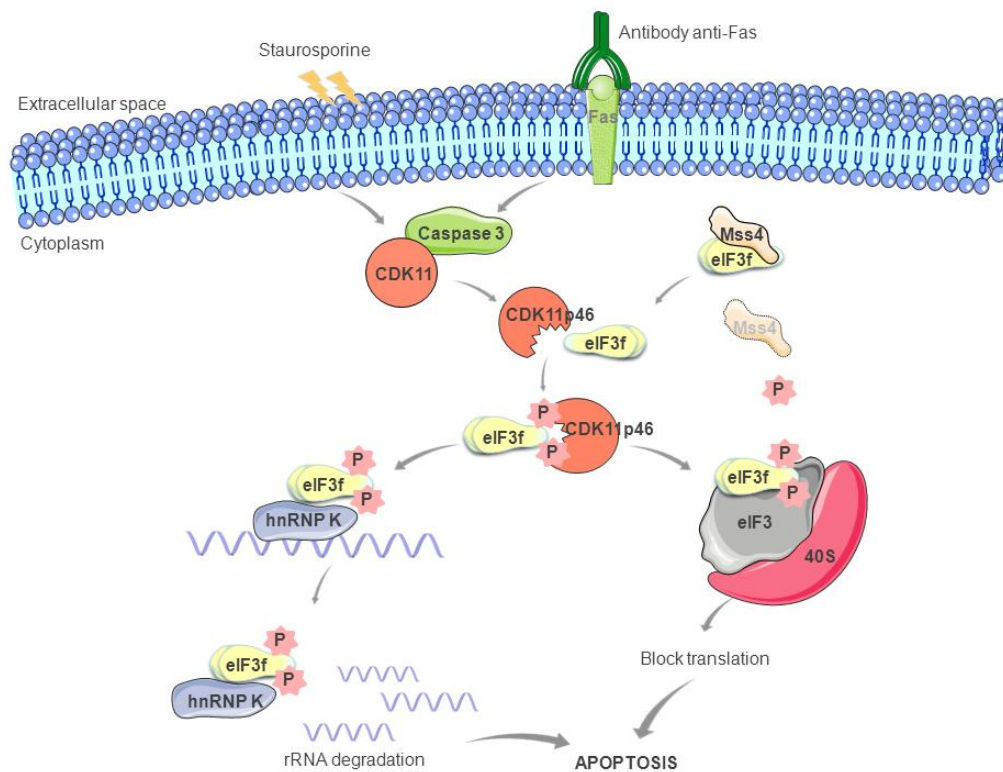


Figure 2. Schematic representation of eIF3f interactions during apoptosis. Upon apoptotic stimulation, CDK11p110 is cleaved by caspase 3 to generate a CDKp46 that interacts strongly with the Mov34 domain of eIF3f and phosphorylates it at Ser46 and Thr119. According to Wen *et al.*' hypothesis (left side), phosphorylated eIF3f interacts with hnRNP K and promotes rRNA degradation by interfering with rRNA protective function of hnRNP. According to Walter *et al.*' hypothesis, under normal conditions (right side) Mss4 is tightly bound to eIF3f, inhibiting its phosphorylation and subsequent association with eIF3 protein complex and pro-apoptotic functions. After prolonged stress-induced apoptosis, Mss4 is downregulated, leading to a release of eIF3f, which is phosphorylated by the CDK11p46 kinase and translation initiation results inhibited.

Thr119 is located in the Mov34 domain of eIF3f, which is important for both the translational inhibitory function and the eIF3f interaction with CDK11p46. The biological consequence of the phosphorylation of eIF3f during apoptosis is not completely known. The phosphorylation status of eIF3f can significantly influence its association with eIF3 core complex and its function in the regulation of translation and apoptosis [60]. Considering that the phosphorylations on Ser46 and Thr119 have been detected in cells undergoing apoptosis, it has been postulated that eIF3f may be involved in the inhibition of the protein synthesis when phosphorylated, acting as a downstream death executor.

4.3.2. eIF3f expression levels in cancer and apoptotic cells

The biological role of eIF3f in translation and apoptosis in tumor cells has been investigated by Shi *et al.* [39, 53-56, 60]. Transcriptome analysis has shown that endogenous eIF3f expression level changes from one cell type to another and from normal to tumor cells [55]. Furthermore, the eIF3f protein level also increases during apoptosis with maximal expression at 36 hours after stimulation [55].

Using a cancer profiling array and real-time reverse transcription PCR, eIF3f transcript levels have been shown to be downregulated in most human tumors relative to matched normal tissues [55]. In particular, 100% of pancreas and vulva tumors, 90% of breast tumors, 71% of melanomas, and 70% of ovary and small intestine tumors showed a significant decrease of eIF3f expression [55]. Using loss of heterozygosity and gene copy number analyses, it has been demonstrated that there is an allelic loss of *eIF3f* gene in human melanoma and in pancreatic cancer cells [53, 54]. No mutations are responsible for the decreased eIF3f expression in these two cancer types [53, 54].

The molecular mechanism by which eIF3f protein expression decreases, contributing to cancer development, is unclear. One possible explanation is that the decrease of a negative regulator of translation like eIF3f may lead to an increased eIF3 activity, which in turn stimulates translation of specific mRNAs encoding for proteins involved in cell proliferation [53, 54]. eIF3f was also found to interact with mTOR and S6K1. The decrease of eIF3f expression may deregulate the function of mTOR pathway, which in turn leads to increased translation of oncogenic proteins and malignant transformation [53, 54]. Finally, eIF3f is

ubiquitously expressed in all tissues and is associated both with subpolysomal particles and with polysomes. eIF3f is localized both at cytoplasmic and at nuclear level according to the cell cycle phase and is able to associate to different sub-fractions of eIF3 complex. Probably, these various multiproteins complexes are implicated in the role of eIF3f in discriminating the mRNAs to be translated into normal cells and may also reflect the deregulation of the translational pathway in tumor cells.

4.3.3. eIF3f overexpression in cancer inducing apoptosis

Enforced expression of eIF3f inhibits translation, cellular growth and proliferation, and induces apoptosis in melanoma and pancreatic cancer cells [53-55]. Ectopic expression of eIF3e and eIF3f subunits in stably transfected NIH3T3 fibroblasts also inhibits cell growth and proliferation [48]. Western blot analyses have demonstrated that eIF3f induces apoptosis in caspase 3/7 and Bcl-2, Bax or Bcl-XL independent manner [55]. It is still unclear whether decreased eIF3f expression is the cause, rather than the consequence, of malignant transformation. Knockdown of endogenous eIF3f by siRNA attenuates apoptosis in melanoma cells after treatment with staurosporine as apoptotic agent [55]. Wen *et al.* have stably knocked down endogenous eIF3f in normal human pancreatic ductal epithelial cells (HPDE) and have observed increased cell proliferation, clonogenicity, apoptotic resistance, survival, resistance to chemotherapy drug, mesenchymal morphology and migration. eIF3f-silenced cells also show an increased cell size, nuclear pleiomorphism, aneuploidy, and cell cycle abnormality [56]. By using an ex-vivo 3D-cell culture system, they have also shown that eIF3f-silenced HPDE cells form more irregular masses with abnormal architecture and polarity (recapitulating malignant tumors *in vivo*), while control cells develop into a single-layer epithelial hollow spheres (resembling normal pancreatic ductal structure *in vivo*). eIF3f-silenced HPDE cells proliferate in an anchorage independent manner. These observations confirm that eIF3f is an important negative regulator of cell growth and proliferation and the decrease of its expression contributes to tumor cells' evading apoptosis via upregulation of protein synthesis [56].

4.3.4. A link between translation initiation and rRNA degradation

Besides the hypotheses described above to explain how decreased eIF3f may contribute to cancer development, a further model linking the translation initiation factor and rRNA degradation has been proposed by Wen *et al.* [56]. This model finds its basis in the observation that eIF3f-transfected cancer cells show a significant decrease of 60S and 80S ribosomes and 60S/40S ribosomal subunit ratio, and an increase in RNA absorbance during apoptosis [55]. It is still unclear whether the reduced 60S ribosomal subunit contributes to or is a result of apoptosis. The increasing of the absorbance is correlated to an increased amount of rRNA degradation, which contributes to apoptotic process [55]. The major rRNA degraded in eIF3f-transfected cells is the 28S rRNA followed by the 18S rRNA in minor rate [55]. How exactly eIF3f induces rRNA degradation is unclear. However, as shown for other eIF3 subunits, the translation may be inhibited by specific eIF3f binding to RNAs and their consequent degradation [29]. The discovery that eIF3f can interact with the heterogenous nuclear ribonucleoprotein K (hnRNP K) during apoptosis in melanoma and HPDE cells, corroborates this model [56]. hnRNP K is a RNA-binding protein responsible for the maintenance of RNA stability by binding to the 3'-UTR of the mRNAs. Yeast three-hybrid screens and RNA pull-down assays have proven that hnRNP binds to 18S and 28S rRNA [72]. In human cells dissociation of hnRNP from rRNA contributes to rRNA degradation during apoptosis [56]. However, whether hnRNP K regulates rRNA degradation is not known. Considering that hnRNP K protects rRNA from degradation whereas eIF3f promotes this process [56], Wen *et al.* have proposed that eIF3f may interact with hnRNP K during apoptosis and promotes rRNA degradation by interfering with its rRNA protective function (Figure 2). In other words, eIF3f sequesters hnRNP K and inhibits its binding to rRNA, which leads to increased rRNA degradation and attenuated translation.

The rRNA synthesis and degradation in normal cells needs to be perfectly balanced in order to maintain homeostasis of protein synthesis. Under physiological conditions eIF3f competes with rRNA for hnRNP K binding. This contributes to the maintenance of the homeostasis of rRNA level and translation in cells. Increased eIF3f expression contributes to apoptosis via hnRNP K sequestration and increased rRNA degradation. Loss of eIF3f contributes to tumorigenesis via the increased binding of hnRNP K to rRNA and an increased rRNA level. Currently, it is still unclear where and how normal human rRNA is degraded

during various physiologic or pathologic cellular processes, however eIF3f might play a role in mRNA and rRNA degradation.

4.3.5. Mss4: the neutralizing agent of eIF3f translation inhibitory effect

Starting from an investigation on the regulatory properties of the mammalian suppressor of Sec4 (Mss4) in response to cellular stress, Walter *et al.* have recently demonstrated that this protein can act as an anti-apoptotic agent by direct binding to eIF3f [73]. High expression levels of Mss4 protein are beneficial for cells, as they protect cells from stress-induced apoptosis. The expression of Mss4 is strongly regulated by stress stimuli at both transcription and post-transcriptional level: it is upregulated at early stages of stress stimulation, but it declines with prolonged stimulation. Yeast two-hybrid screens have indicated that the most abundant Mss4 interaction partner is eIF3f and studies on A7 melanoma cells have shown that Mss4 neutralizes the translation inhibitory effect of eIF3f [73]. Thus, Mss4 is probably involved in regulation of protein translation and its association to eIF3f protects cells from apoptosis. In other words, eIF3f is a negative regulator of translation: its overexpression makes apoptosis easier, whereas its downregulation has the opposite effect. Under normal conditions, Mss4 is tightly bound to eIF3f, inhibiting its phosphorylation and subsequent association with eIF3 complex. At early stages of stress stimulation, increasing amounts of Mss4 protein efficiently bind the eIF3f preventing its interaction with CDK11p46. When Mss4 proteins in cells drop down owing to ongoing stress, the CDK11p46 can phosphorylate the released eIF3f. The translation initiation is inhibited and apoptosis is activated (Figure 2).

4.4. Atrophy versus hypertrophy: the importance of eIF3f

4.4.1. Hypertrophy

In muscle cells also, the regulation of protein synthesis rate has an important role in the control of cell growth. Muscular hypertrophy and atrophy are two opposite and mechanistically linked phenomena regulating muscle cell size, finely determined throughout a balance between new protein accumulation and degradation of pre-existing proteins [74]. A major mediator of skeletal muscle hypertrophy is the serine/threonine-specific protein kinase Akt1. Two major downstream branches of the Akt pathway, which are relevant to muscle

hypertrophy, are the mTOR pathway, which is activated by Akt, and glycogen synthase kinase 3 β , which is blocked by Akt. Both pathways control the protein synthesis [74].

The kinase mammalian target of rapamycin (mTOR) has recently emerged as a key regulator of mammalian cell growth that integrates signals from growth factors, nutrients, and energy status to control protein synthesis and other cell functions [75, 76]. As the name implies, mTOR is selectively inhibited by rapamycin, a drug used as an immunosuppressant in organ transplantation. The role of mTOR in muscle growth has been demonstrated by *in vivo* studies showing that rapamycin blocks overload hypertrophy and regenerating muscle growth [77, 78]. The effect of mTOR on the translation machinery and protein synthesis is executed by phosphorylation of the ribosomal protein S6 kinases and of 4E-BP1, a repressor of the cap-binding protein eIF4E [79, 80]. mTOR and S6K1 maneuver on and off the eIF3 translation initiation complex in a signal-dependent fashion [79]. In fact, the eIF3 complex acts as a scaffolding platform that associates with mTOR and S6K1 in a growth-factor and rapamycin-sensitive manner. Under normal conditions, S6K1 is bound to the eIF3 complex, whereas mTOR association is greatly reduced. Following an activating signal, such as insulin stimulation, mTOR is recruited to the eIF3-preinitiation complex, leading to phosphorylation of the bound and inactive S6K1. Phosphorylated S6K1 is released from eIF3 complex, and interacts with PDK1, which promotes a second phosphorylation of S6K1. Activated S6K1 then phosphorylates eIF4B, which is then recruited to the eIF3-preinitiation complex and promotes the recruitment of other initiation factors and the translation of mRNAs encoding proteins involved in muscle hypertrophy (Figure 3, upper panel) [79]. Thus, eIF3, mTOR, and S6K1 coordinate the assembly of a translation initiation complex with enhanced translational capacity under conditions of nutrient and energy sufficiency. The understanding of how this assembly is controlled is still incomplete. However a physical and functional link exists between these proteins. Yeast two-hybrid screens have revealed that both mTOR [80] and S6K1 [79] physically interact with the f subunit of eIF3. Particularly, the inactive hypophosphorylated form of S6K1 physically associates with the Mov34 domain of eIF3f [81]. Thus, mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein/protein interchange and might control the function of eIF3 [80].

4.4.2. Translational regulation of eIF3f

The mRNA encoding for the eIF3f factor contains at 5'-end a relatively short stretch of 6-12 pyrimidines, called terminal oligopyrimidine (TOP) sequence, and consequently its mRNA is known as TOP mRNA [82]. The TOP sequence serves as a cis-acting element necessary for a growth-associated translation regulation [83]. The translational control of TOP mRNAs is used by cell to coordinate the synthesis of different ribosomal proteins, elongation factors, and several other proteins associated with the assembly or function of the translational apparatus [83]. Interestingly, among the initiation factors only eIF3e, eIF3f and eIF3h exhibit the characteristics of TOP mRNAs [82]. A polysome mRNA analysis on HeLa, HEK293 and NIH3T3 cells has revealed that eIF3e, eIF3f and eIF3h mRNAs are mainly associated with subpolysomal particles in quiescent or growth-arrested cells but mostly associated with polysomes in growing cells [82]. Consequently, the fact that these three eIF3 subunits are encoded by TOP mRNAs is considered relevant for their role in the growth-associated translation regulation.

It is classically known that mTOR Complex 1 (mTORC1) regulates cell growth and proliferation through modulation of protein synthesis in response to growth factors, hormones and amino acids [84]. In a recent study, it has been demonstrated that the mTORC1 kinase specifically controls the translation of TOP mRNAs through the eIF4E-binding proteins (4E-BPs) and the eIF4G1 initiation factor [85]. In eIF4E-dependent initiation, mTORC1 phosphorylates the 4E-BPs promoting their dissociation from the cap-binding protein eIF4E. Active eIF4E then associates to eIF4G1, which interacts with eIF3 resulting in the assembly of the 43S pre-initiation complex on the mRNA [75]. When mTORC1 is inactivated by rapamycin or Torin1, dephosphorylated 4E-BPs bind eIF4E and thereby prevent its association with eIF4G1 and the consequent activation of TOP mRNA translation [85].

In 2010, Csibi *et al.* have reported that the decreased activity of mTORC1 kinase is correlated with the degradation of eIF3f and the accumulation of unphosphorylated forms of S6K1 during muscle atrophy [86]. In contrast, during terminal muscle differentiation the amount of eIF3f increases as well as mTORC1, leading to an increase in S6K1 activation and phosphorylation of rpS6 and 4E-BP1 [86]. They also observed that rapamycin treatment destabilizes the mTORC1/eIF3f interaction in differentiated myotubes and ablation of eIF3f

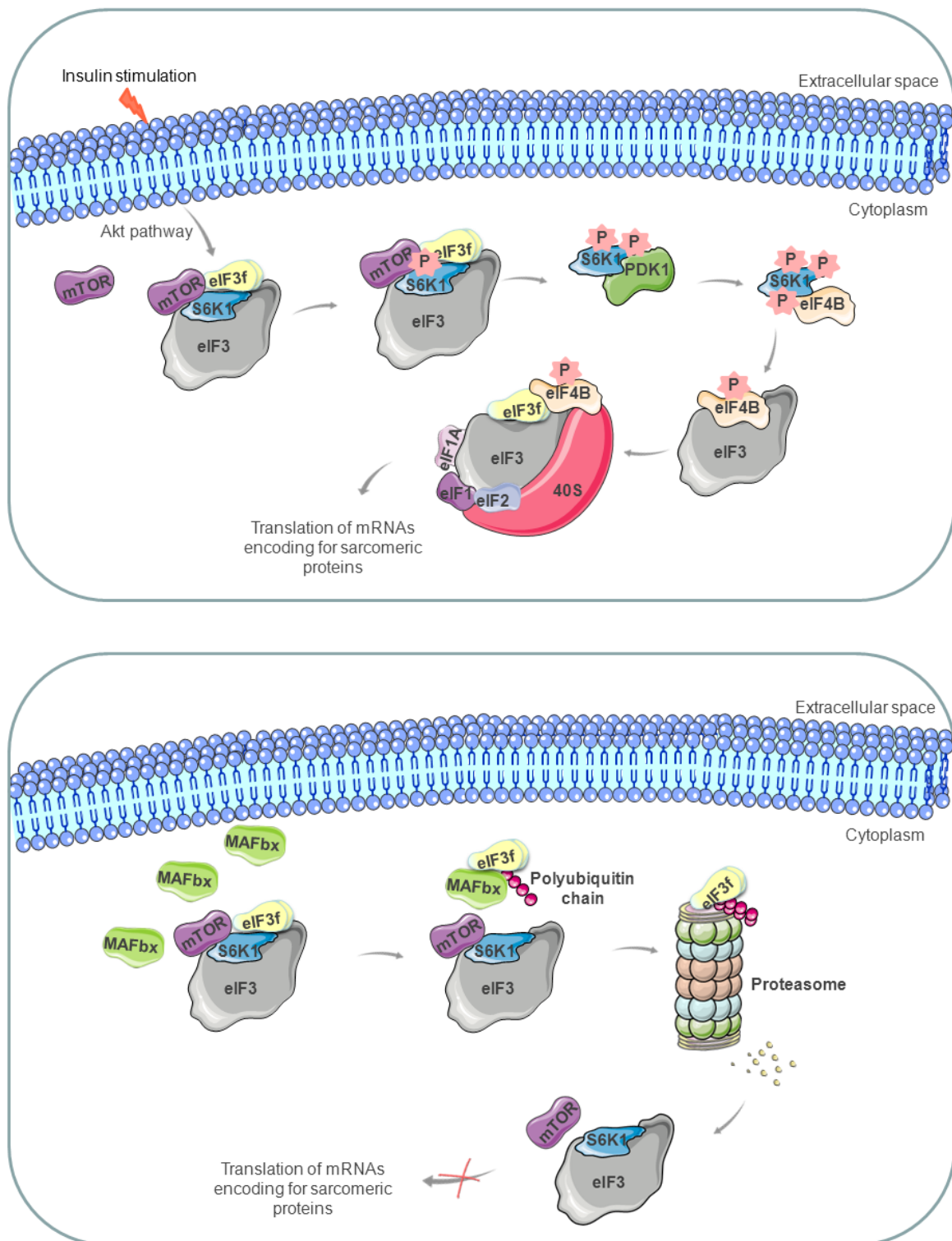


Figure 3. Schematic representation of the intracellular signals characterizing the hypertrophic (upper panel) and atrophic (lower panel) pathways in muscle cells. During hypertrophy, mTOR phosphorylates S6K1, which is released from eIF3 complex, and phosphorylated again by PDK1. Activated S6K1 phosphorylates eIF4B, which promotes the recruitment of other initiation factors and the translation of mRNAs encoding proteins involved in muscle growth. During atrophy, the ubiquitin ligase MAFbx is upregulated and S6K1 is accumulated in its inactive hypophosphorylated form. By binding the Mov34 domain, MAFbx transfers polyubiquitin chains on eIF3f and promotes its degradation by the proteasome and block the synthesis of proteins involved in muscle cell growth.

in muscle cells prevents mTORC1 activity, phosphorylation of S6K1, rpS6 and 4E-BP1 [86]. Thus, in muscle cells the eIF3f translation may be regulated in an mTORC1 dependent manner.

4.4.3. Atrophy

Atrophy is a decrease in cell size mainly caused by a loss of organelles, cytoplasm and proteins. A major contribution in understanding muscle atrophy comes from the pioneering studies on gene expression profiling performed independently by Gomes and Bodine [87, 88]. The idea to compare gene expression in different models of muscle atrophy leads to the identification of a subset of genes that are commonly up- or down- regulated. Since all the diseases used for the experiments of microarray (*i. e.*, diabetes, cancer cachexia, chronic renal failure, fasting, and denervation) have muscle atrophy in common, the up or down genes are believed to regulate the loss of muscle components and are called atrophy-related genes or atrogenes [89]. Together, these findings indicate that muscle atrophy is an active process controlled by specific signaling pathways and transcriptional programs [74]. Furthermore, the two most induced genes encode for two muscle-specific ubiquitin ligases, atrogin-1/MAFbx and MuRF1, that are upregulated in different models of muscle atrophy and are responsible for the increased protein degradation through the ubiquitin-proteasome system [87, 88]. These two genes are actually the best markers for muscle atrophy and could be considered as master genes for muscle wasting.

Ectopic expression of MAFbx in myotubes leads to atrophy and appears to be essential for accelerated muscle protein loss [90]. Interestingly, during atrophy MAFbx physically interacts with the f subunit of eIF3 via its Mov34 domain [91], leading to its ubiquitination and its degradation by the proteasome (Figure 3, lower panel) [90]. The silencing of MAFbx expression by small hairpin RNA interference prevents eIF3f degradation in myotubes undergoing atrophy [91]. In C2C12 myotubes that undergo atrophy, MAFbx accumulates in the nucleus and triggers the translocation of eIF3f from the cytoplasm to the nucleus. The translocation of the f subunit between the two compartments may represent a mechanism for regulating its activity during atrophy.

Interestingly, eIF3f can itself act as an enzyme by deubiquinating the transmembrane receptor Notch1 [92]. Notch1 is involved in a highly conserved signaling pathway essential for development and is monoubiquitinated during its activation. To enter the nucleus and fulfill its transcriptional functions, this receptor needs to be deubiquitinated. The f subunit acts as a positive regulator of Notch signaling. The activated form of Notch is able to interact with eIF3f only in the presence of the E3 ubiquitin ligase Deltex [92]. It remains to be elucidated whether eIF3f works as a deubiquinating enzyme outside the translation complex or in association with the whole translation initiation complex.

4.4.4. The role of eIF3f in atrophy and hypertrophy

By using an inducible protein expression system, it has been demonstrated that the genetic activation of eIF3f is sufficient to block atrophy and induces a massive hypertrophy. This cellular growth is associated with an increase of sarcomeric proteins but not the muscle regulatory factors such as MyoD and/or myogenin [91]. eIF3f is upregulated during terminal differentiation of skeletal muscle and absent in the undifferentiated embryonic rhabdomyosarcoma. Hence, in skeletal muscle eIF3f can act as a ‘translational enhancer’ that increases the efficiency of the structural muscle protein synthesis leading to muscle hypertrophy *in vitro* and *in vivo*. These findings are consistent with previous suggestions that overload-induced hypertrophy is due to an increase in translational capacity and/or translational efficiency [93, 94]. Thus, eIF3f plays an important role in controlling muscle mass and size.

On the contrary the loss of eIF3f, due to MAFbx mediated ubiquitination, induces atrophy in normal myotubes. Proteins destined for degradation are marked by covalent linkage with a chain of ubiquitin molecules on lysine residues for further degradation into short peptides by the 26S proteasome. Deletion studies, focused on the identification of the lysine residues marked by MAFbx, have shown that the target for proteasomal degradation is the C-terminal domain of eIF3f. This domain contains 6 C-terminal lysine residues that serve as ubiquitination sites. The mutation of these 6 lysine in arginine residues (mutant K5-10R) does not suppress the MAFbx-eIF3f interaction nor its activity. Thus, the C-terminal domain finely regulates eIF3f activity in skeletal muscle [90]. When levels of eIF3f are maintained by electroporation of the eIF3f K5-10R mutant into adult fibers, muscles are protected from

atrophy [90]. The eIF3f mutant K5-10R induces a strong hypertrophic phenotype in cellulo and *in vivo*, and its overexpression provokes an enhanced ability in avoiding skeletal muscle atrophy [90].

The ubiquitination of eIF3f on C-terminal lysine residues may serve two functions: addressing eIF3f degradation specifically by MAFbx during skeletal muscle atrophy and regulating the translation of specific pools of mRNAs of key sarcomeric proteins [90].

Hence, atrophy and hypertrophy processes communicate each other via the translation regulator eIF3f [81]. Its Mov34 domain is necessary for both S6K1 and MAFbx binding. In particular, muscles undergoing atrophy accumulate the inactive hypophosphorylated form of S6K1, stimulating the MAFbx-mediated degradation of eIF3f (Figure 3, lower panel). Whether MAFbx interacts with free eIF3f or with eIF3f molecules bound to S6K1, still remains unclear.

5. Conclusion

In the light of the eIF3f properties illustrated above, a global analysis of its interactome is necessary. Two opposite features of eIF3f have emerged by analyzing the data available in literature. This factor is able to act both as a ‘negative repressor’ of translation initiation [39], and as a ‘translational enhancer’ [91], both as a ‘downstream death executor’ in cancer cells [55] and also as a ‘cell growth enhancer’ in muscle cells [91]. The loss of eIF3f seems to contribute to tumorigenesis whereas its ectopic expression is sufficient to restore apoptosis in cancer cells. Interestingly, in normal myotubes the genetic repression of eIF3f induces atrophy whereas genetic activation of eIF3f is sufficient to induce hypertrophy.

The same protein seems to act in two different ways according to the cell type considered. It is not clear whether the endogenous eIF3f protein rate is the cause or the effect of this behaviour. However, a cell-type specific role is believed for eIF3f.

Several eIF3f interacting partners have been identified. During the apoptotic pathway, eIF3f is activated by CDK11p46, interacts with hnRNP K for RNA degradation, and is neutralized by Mss4. During the muscle cell growth, eIF3f interacts with mTOR/S6K1 to promote the assembly of the translation initiation complex and is degraded by MAFbx. A positive role in the activation of Notch signaling pathway has been also reported.

Moreover, two different types of eIF3 complexes (nuclear and cytoplasmic) have been found in the cell, suggesting that eIF3f could shuttle between the cytoplasm and the nucleus in a cell cycle-dependent manner.

All these data confirm that eIF3f has a multileveled control of multiple functions in the cells, outside its usual function in translation. Keeping it in mind, targeting eIF3f may be a strategy to reorganize different intracellular pathways and alter the basis of the balance between cell proliferation and apoptosis. Thus, eIF3f represents a lead candidate to use for biotherapeutic applications both for inhibiting the growth of cancer cells or muscle atrophy and thus preventing its progression into irreversible cachexia.

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Recent updates

Since the publication of this review, other studies on the function of the eIF3f subunit in various cellular functions have recently emerged.

For example, it has been confirmed that eIF3f serves as a docking site for S6K to phosphorylate other eIF3 subunits, and that the interaction eIF3f-S6K1 controls the subsequent interaction of Paip1 (poly(A)-binding protein-interacting protein 1) with eIF3 complex (Martineau *et al.*, 2014). This study strengthens the role of the f subunit in the control of translation initiation and its translation promoting activity. The same eIF3f function emerges also from another study conducted on the deleterious effects of chronic alcohol consumption in heart muscles (Lang *et al.*, 2014). This study has demonstrated that chronic consumption of alcohol induced a decrease of protein synthesis in cardiac cells and this effect is associated to reduced activity of mTOR, S6K1 and eIF3f (Lang *et al.*, 2014).

It has also been recently identified the molecular ‘stabilizer’ of eIF3f in murine initiation translation complex. The eIF3m subunit maintains eIF3 integrity by stabilizing the core subunits eIF3f and eIF3h (Zeng *et al.*, 2013). In particular, when bound to eIF3m, the eIF3f subunit is protected by degradation through subcomplex formation (Zeng *et al.*, 2013).

Finally, a novel interacting partner has been identified in human cells. The N-terminal region of eIF3f (amino acids 1-113) has been shown to interact with hMSH4 protein which participates in the DNA repair process (Chu *et al.*, 2013). In response to radiation-induced DNA damage in human cells, eIF3f stabilizes hMSH4 and promotes an early G2/M arrest, thus impeding cell survival (Chu *et al.*, 2013). These data confirm that the contribution of eIF3f to various cellular processes can be determined by the interaction with different molecular partners.

Preface part II

The expression eIF3f subunit is downregulated both at the protein and mRNA levels in different cancer cells (Doldan *et al.*, 2008a; Doldan *et al.*, 2008b). The ectopic expression by transient gene transfection inhibits overall cellular protein synthesis, induces degradation of 28S rRNA, inhibits cell growth and induces apoptosis in melanoma and pancreatic cancer cells (Doldan *et al.*, 2008a; Doldan *et al.*, 2008b; Shi *et al.*, 2006; Wen *et al.*, 2012).

Starting from these observations, we reasoned that a powerful protein therapy approach for cancer treatment could consist in the replenishment of the intracellular levels of eIF3f proteins. The second part of this chapter describes the development of an intracellular protein transfer system, based on the generation of eIF3f recombinant proteins containing the MD₁₁ cell-penetrating sequence. The usefulness of this system consists in direct availability of the cargo proteins upon internalization given the endocytosis-independent mechanism of uptake.

For delivery experiments, the human wild-type (wt) eIF3f protein and three murine mutants were used: a first truncation mutant containing the N-terminal part of eIF3f (amino acids 1-91), a second truncation mutant containing the Mov34 domain and the eIF3f C-terminal end (amino acids 92-361) and a third mutant (described in (Csibi *et al.*, 2009)), with Lys-to-Arg mutations in C-terminus, capable to resist to polyubiquitination and proteasome degradation. Human and murine wt sequences show 92% identity. Murine sequences were chosen both with the long-term prospect to study the effect of recombinant proteins in mouse models and to explore afterwards this protein transfer technology in muscle atrophy treatment.

The initial part of the protein delivery study has regarded the production and the purification of the recombinant fusion proteins. This process has demanded an intense study (not detailed in the following sections) on the optimization of protein expression conditions in *E. coli* system and of the purification methods used for recovering the proteins of interest. As mentioned in Chapter I preface, some detected issues are the tendency of the fusion proteins to incorrectly fold during the production process (inclusion bodies) and to aggregate after purification. To improve the expression of active soluble proteins, strategies like the modification of the cell growth medium or the use of low expression temperatures were used.

For purification strategies, the combination of different methods was tested and a screening of solubility using modified buffer compositions was performed. The effect of additives (*e. g.* sugars, detergents, amino acids, salts, polyols, polysorbate) was also analyzed with the aim of enhance protein solubility and stability. Notwithstanding, the soluble proteins were purified in low yields. Therefore, some of the recombinant proteins were purified under denaturing conditions and subsequently refolded by dialysis. It should be noted that this method allows the recovering of high yields of functional and pure recombinant proteins (Becker-Hapak *et al.*, 2001; Gonzalez-Montalban *et al.*, 2007). It has been reported that, even though recombinant proteins are not correctly folded after purification, they can be refolded by chaperones upon internalization and that they transduce more efficiently into cells than correctly folded proteins (Nagahara *et al.*, 1998; Schwarze *et al.*, 1999). In such cases, urea-denatured cell-penetrating recombinant proteins have been successfully used for *in vitro* and *in vivo* applications (Nagahara *et al.*, 1998; Schwarze *et al.*, 1999).

The following part of the chapter describes the screening of the MD₁₁-eIF3f recombinant protein therapeutic effectiveness in a panel of tumor and normal lines. The *in vitro* evaluation of the antitumor effect of recombinant proteins has led to the identification of three cell lines (murine and human melanoma and human colorectal carcinoma), in which the replenishing of the eIF3f levels efficiently induces the tumor-cell death.

This work has allowed to demonstrate that (i) the MD₁₁ cell-penetrating peptide can efficiently mediate the non-invasive internalization of proteins of therapeutic interest into several mammalian cell lines, (ii) the cargo proteins have direct access to cell cytoplasm resulting in a rapid response to protein treatment, (iii) the MD₁₁- based eIF3f transfer system may represent a powerful strategy for cancer treatment.

Although highly efficient in mediating the *in vitro* cellular uptake of different proteins into many cell lines, the MD₁₁ cell-penetrating peptide lacks of cell specificity. Indeed, the ubiquitination resistant mutant and the NF truncation show strong but cell-unspecific killing properties, while the wt protein does not induce death in normal cells. Thus, the choice of the coupled therapeutic protein could represent a strategy for bypassing the lacking of cell targeting of MD₁₁ peptide.

MD₁₁-mediated delivery of recombinant eIF3f induces melanoma and colorectal carcinoma cell death

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Short title: Intracellular delivery of a cell-penetrating eIF3f

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1. Abstract

The f subunit of the eukaryotic initiation factor 3 (eIF3f) is downregulated in several cancers and in particular in melanoma and pancreatic cancer cells. Its enforced expression by transient gene transfection negatively regulates cancer cell growth by activating apoptosis. With the aim to increase the intracellular level of eIF3f proteins and activate apoptosis in cancer cell lines, we developed a protein transfer system composed of a cell-penetrating peptide sequence

fused to eIF3f protein sequence (MD₁₁-eIF3f). To determine whether exogenously administered eIF3f proteins were able to compensate the loss of endogenous eIF3f and induce cancer cell death, we analyzed the therapeutic action of MD₁₁-eIF3f in several tumor cells. We identified four cell lines responsive to eIF3f-treatment and we evaluated the antitumor properties of the recombinant proteins using dose- and time- dependent studies. Our results demonstrate that this protein delivery approach represents an innovative and powerful strategy for cancer treatment.

2. Introduction

Protein therapy is one of the most direct and safe approach for treating some of the most difficult-to-treat diseases, by which the missing or defective protein is produced by recombinant methods and delivered directly into human cells [1]. As only compounds within a narrow range of molecular size and polarity passively penetrate into cells, the plasma membrane represents a major limit for the delivery of peptides or proteins. In recent years, substantial progresses have been made to find and design novel therapeutic technologies for improving the cellular entry of hydrophilic macromolecules with cytoplasmic or nucleic targets. Cell-penetrating peptides (CPPs) or Protein Transduction Domains (PTDs) are short peptides able to gain access to cytoplasm and subcellular compartments by different mechanisms, including endocytosis, and to promote the intracellular delivery of different cargoes [2]. CPP-mediated delivery of functional proteins or peptides has been extensively employed both for studying cellular processes and for developing novel therapeutic macromolecules [3]. A novel cell-penetrating peptide deriving from the Epstein-Barr virus (EBV) ZEBRA transcription factor has been recently described by our group [4]. The minimal amino acid region implicated in cellular uptake spans residues 178-220 of full-length ZEBRA protein (MD₁₁), and is able to translocate high molecular weight proteins in an endocytosis-independent mechanism, allowing the internalization of cargo proteins in fully biologically active form.

Eukaryotic gene expression is a process mainly regulated at the levels of gene transcription and protein synthesis. Minimal deregulation of protein synthesis can lead to uncontrolled cell growth and cancer formation. The translation process is largely regulated at initiation level by 12 different eukaryotic initiation factors (eIFs). The involvement of several

eIFs in pathogenesis and cancer development has been already reported [5]. In particular, several lines of evidence suggest that the eukaryotic initiation factor 3 (eIF3) contributes to tumorigenesis. This complex is composed of 13 subunits (designated eIF3 a-m). Amongst the 13 subunits, overexpression of subunits eIF3-a, -b, -c, -h, -i and -m has been detected in several different solid tumors and in different cancer cell lines [6-9]. Two other subunits eIF3e and eIF3f are downregulated in many human tumors, and in particular the f subunit expression is significantly decreased in 100% of pancreas and vulva tumors, 90% breast tumors, 71% melanomas and 70% of ovary and small intestine tumors [10].

eIF3f belongs to the Mov34 protein family containing the MPN motif [11] and acts as a negative regulator of translation inhibiting both cap-dependent and cap-independent translation [12]. The exact molecular mechanisms, by which eIF3f expression decreases contributing to cancer development, are still unclear. It is likely that the loss of eIF3f in cancer cells induces increased eIF3 activity, which in turn stimulates translation of specific mRNAs encoding proteins involved in cell proliferation [13, 14]. In melanoma and pancreas cancer cells, eIF3f exhibits a tumor-suppressive role as its enforced expression by gene transfection negatively regulates cancer cell growth by activating apoptosis [10, 12-14].

However, in proliferating myoblasts eIF3f protein is barely detectable, but is dramatically upregulated during terminal differentiation and maintained in adult skeletal muscle [15]. Interestingly, the genetic repression of eIF3f in normal myotubes induces atrophy while genetic activation is sufficient to induce hypertrophy through modulation of protein synthesis via the mTORC1 pathway [16, 17]. Therefore, eIF3f plays a central role in the control of muscle mass and size and a cell-type-specific role is believed to exist for eIF3f [18].

In this study, we hypothesize that increasing of eIF3f protein intracellular level by protein transduction could represent a powerful approach to cancer treatment. To test this hypothesis, we produced fusion recombinant proteins (MD₁₁-eIF3f) in which the eIF3f sequence is directly fused to the MD₁₁ cell-penetrating peptide, previously isolated and characterized in our laboratory [4]. We performed experiments to determine whether exogenously administered MD₁₁-eIF3f recombinant proteins were able to compensate the loss of endogenous eIF3f and induce a cellular death by inhibiting the protein synthesis process.

We engineered two eIF3f truncations (MD₁₁-AN and MD₁₁-NF) and an ubiquitination-resistant mutant (MD₁₁-K510R) [19]. The therapeutic efficiency of these proteins was screened in several tumor cells. We identified four eIF3f-treatment respondent cell lines (B16 and Colo857 melanoma cells; HCT116 p53^{+/+} and HCT116 p53^{-/-} colorectal carcinoma) and we evaluated the antitumor properties of the recombinant proteins using dose- and time-dependent studies. Our results demonstrate that the MD₁₁ peptide can efficiently deliver functional eIF3f in tumor cells, and that this protein delivery system represents a potentially powerful tool for treating melanoma and human colorectal carcinoma.

3. Materials and methods

3.1. Expression vectors of MD₁₁-eIF3f fusion proteins

The coding sequence of full-length human eIF3-f was generated by PCR using the forward primer 5'-TAACGCCTCGAGGCCACACCGGCGGTACCAGTAAGTGCTCCTCCG-3' and the reverse primer 5'-ATTCTTATGGATCCTTACAGGTTTACAAGTTTTTCATTG-3', which contain at 5' ends XhoI and BamHI restriction sites respectively. Two truncation mutants of murine eIF3f were generated: the mutant AN extending from nucleotide 1 to 91 and the mutant NF extending from nucleotide 91 to 361 using the following primers:

eIF3f₁₋₉₁ forward: 5'- TAACGCCTCGAGGCTTCTCCGGCCGTACCG-3';

eIF3f₁₋₉₁ reverse: 5'- CGCGGATCCTCAGAAAGGCCCGGGAGG-3';

eIF3f₉₂₋₃₆₁ forward: 5'- TAACGCCTCGAGCCGGGCGGCCGCGTGG-3';

eIF3f₉₂₋₃₉₁ reverse: 5'- CGCGGATCCTCACAGGTTTACAAGTTTCTCGT-3'.

The murine ubiquitination resistant mutant K5-10R sequence, previously described in [19], was amplified using eIF3f₁₋₉₁ forward primer and the reverse primer 5'-CGCGGATCCTCACAGGTTTACAAGTCTCTC-3'. Downstream fusion of the amplified fragments to ZEBRA-MD₁₁ sequence was performed as described in [20]. After amplification, the DNAs of interest were digested with appropriate restriction enzymes and ligated into the optimized pET15b vector containing the sequence of ZEBRA MD₁₁. All cloned plasmid sequences were verified by sequencing.

3.2. Expression and purification of recombinant fusion proteins

For optimum expression yields, the MD₁₁-AN, MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R recombinant fusion proteins were expressed in *E.coli* after induction at OD_{600nm}=0.8 with IPTG for 18 h at 16 °C in following conditions:

Protein	<i>E. coli</i> bacterial strain	[IPTG] mM	Additive
MD ₁₁ -AN	BL21-CodonPlus (DE3)-RIPL (Stratagene)	0.2	0.4M sucrose
MD ₁₁ -NF	<i>E. coli</i> BL21 (DE3) (Novagen)	0.5	None
MD ₁₁ -wt	BL21-CodonPlus (DE3)-RIPL	0.5	None
MD ₁₁ -K510R	BL21-CodonPlus (DE3)-RIPL	0.2	10g/L glycerol

Bacteria were then harvested by centrifugation at 10000g for 10 minutes at 4°C. After washing with PBS buffer, cell pellets were resuspended in 5mL/g cell pellet of 20mM MOPS pH 7.0, 250mM NaCl, 5% glycerol, 5mM imidazole (lysis buffer) supplemented with complete protease inhibitor cocktail (Roche) and sonicated. The soluble and insoluble fractions (inclusion bodies, IBs) were separated by centrifugation at 13000g for 30 minutes at 4°C.

MD₁₁-AN was purified from the soluble fraction onto a Ni-NTA column (Qiagen) using FPLC liquid chromatography system. Protein of interest was eluted in lysis buffer by a stepwise increase (at 100, 250 and 500mM) of imidazole content.

MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R were purified from inclusion bodies under denaturing conditions (insoluble fraction). As a first step of the purification procedure, IBs were washed in 20mM MOPS pH 7.0, 15mM cysteine, 2M NaCl for 1h at 4°C and centrifuged at 14000g for 30 minutes at 4°C. Two additional washing steps were performed in 20mM MOPS, 15mM cysteine, 250mM NaCl, 2% Triton X-100, and in 20mM MOPS pH 7.0, 15mM cysteine, 250mM NaCl for 1h at 4°C respectively. IBs were then solubilized for 18 hours at 4°C in 20mM MOPS pH 7.0, 15mM cysteine, 250mM NaCl, 8M Urea (solubilization buffer). The samples were centrifuged at 14000g for 30min at 4°C and the supernatants were applied to HisGraviTrap columns (GE Healthcare). Proteins of interest

were eluted at room temperature in solubilization buffer at pH 6.5 by a stepwise increase (at 250, 500mM and 1M) of imidazole content.

The following refolding steps were performed at 4°C. Elution fractions containing purified proteins were dropwise diluted at concentrations ranging from 0.1-0.05 mg/mL in 50mM Na citrate pH 6.1, 2mM MgCl₂, 4mM DTT, 50mM NaCl, 0.5M L-arginine, 0.5% Tween 20 and degassed with N₂ for 20 minutes. After 24 hours of gentle stirring, the samples were concentrated to 5 mL using MWCO 10kDa Amicon centrifugal filter (Millipore) and dialyzed twice against 50mM Na citrate pH 6.1, 2mM MgCl₂, 4mM DTT, 50mM NaCl, 0.05M L-arginine, 0.01% N-lauroylsarcosine. Soluble refolded proteins were recovered by centrifugation at 17000g for 10min. Prior to their use for cellular uptake experiments, MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R purified proteins were dialyzed twice against 50mM Na citrate pH 6.1, 50mM NaCl, 10% glycerol and MD₁₁-AN against 25mM HEPES/KOH pH 7.0, 50mM NaCl and 10% glycerol.

Proteins were separated onto a 12-15% SDS-polyacrylamide gel electrophoresis and analyzed by Coomassie blue staining or by Western-blotting using an anti-His tag antibody HRP-coupled (Sigma, 1:10000 dilution) or using anti-eIF3f antibody (Rockland, 1:1000 dilution) and an anti-rabbit HRP-labeled secondary (GE Healthcare). The yields of purified his-tagged proteins were quantified by BCA Protein Assay Kit according to the manufacturer's instructions (Pierce).

3.3. Cell culture and treatments

Cells were cultured at 37°C in a humidified 5% CO₂ atmosphere incubator in following media:

Cell line	Medium
B16, GL26,	DMEM with stable glutamine and Na pyruvate (PAA, GE Healthcare),
HEK293E, HeLa	10%FBS (PAA, GE Healthcare), 1%Penicillin/Streptomycin (Gibco)
C2C12	DMEM with stable glutamine and Na pyruvate, 15%FBS, 1%Penicillin/Streptomycin

MCF7	DMEM with stable glutamine and Na pyruvate, 10%FBS, 1%Penicillin/Streptomycin, 10µg/mL insulin (Sigma), 0.1mM non-essential amino acids (Gibco)
MCF10-2A	F12 DMEM with stable glutamine and Na pyruvate (Gibco), 10% FBS, 10µg/mL insulin, 0,5µg/mL hydrocortisone (Gibco), 100ng/mL cholera toxin (Sigma), 20ng/mL epidermal growth factor (Sigma) and 1% Penicillin/Streptomycin
HCT116 p53+/+ and HCT116 p53-/-	McCoy's with stable glutamine (PAA, GE Healthcare), 10%FBS, 1%Penicillin/Streptomycin (supplemented with 50µg/mL G418 (Gibco) for p53-/- cells)
Saos-2	McCoy's with stable glutamine , 15%FBS, 1%Penicillin/Streptomycin
LN-229, T98G, U87, Colo857	RPMI-glutamax (Gibco), 10%FBS, 1%Penicillin/Streptomycin
Raji	RPMI-glutamax, 10% FBS, 1mM Na pyruvate (Gibco), 0.1mM non-essential aminoacids, 10µg/mL gentamicin (Gibco)
BJ	DMEM with stable glutamine and Na pyruvate, 10%FBS, 0.1mM non-essential aminoacids

3.4. ⁵¹Cr-release cytotoxicity assays

For cytotoxicity activity, ⁵¹Cr-release assay was used to screening protein-respondent cell lines and to determine time- and dose- dependent kinetics on selected cell lines. From 1 to 3 x10⁶ cells were harvested in complete medium (described above) and then incubated with ⁵¹Cr (Perkin Elmer) at 37°C in 5% CO₂ humidified air for 1 h. Cells were washed with complete medium twice and re-incubated at 37°C for 1 h. Cells were washed with serum-free medium and resuspended to a concentration of 1x10⁵ cells per mL. Aliquots of 100µL of labeled cells were loaded into 96-well plates in triplicate. Saponin (0.1%, Sigma) was added to determine cellular maximum ⁵¹Cr release and serum-free medium was used to determine the spontaneous release. Doxorubicin (1µM, Sigma) was used as toxicity control and protein-free buffers were tested to discard any buffer-linked toxicity.

Recombinant purified proteins were diluted to different concentrations in appropriate serum-free media and aliquots of 100µL were added to cells. After 4 hours protein incubation,

10% FBS was added to cells. The ^{51}Cr incorporated into the cell cytosol by diffusion is released into cell medium in case of protein induced membrane damage. For measurements of ^{51}Cr release, 20 μL of cell-free supernatant were read in a gamma counter (Perkin Elmer).

For cell/line screening assay, 10nM MD₁₁-wt eIF3f and MD₁₁-K510R were tested and measurements were performed 0.5; 1; 2; 3; 4; 8; 18 hours after protein incubation. For dose- and time- dependent study, 2.5; 5; 10 and 50nM of MD₁₁-AN, MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R were used and measurements were collected 0.5; 1; 2; 3; 4; 8; 18 hours after protein incubations.

Percent specific cell death was calculated using the following formula: $(x - SR)/(TR - SR) \times 100$, where x represents the amount of radioactivity released from target cells incubated with proteins, SR represents the amount of spontaneous release of radioactivity from the target cells (target cells incubated with serum-free medium), and TR represents the maximum amount of radioactivity released when target cells are lysed with saponin.

3.5. Protein labeling and intracellular detection

To track the cellular internalization of MD₁₁-AN, MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R, fusion proteins were fluorescently labeled using Alexa Fluor[®] 488 Protein Labeling Kit (Molecular Probes, Invitrogen) according to manufacturer's instructions. For microscopy analysis, B16, Colo857, HCT116 p53^{+/+} and HCT116 p53^{-/-} cells (7×10^4 cells/well) were seeded onto an 8-well Lab-Tek[™] chambered coverglass (Nunc) in complete cell culture media 24h before treatment. After removal of the medium, the cell layers were rinsed twice with DPBS and subsequently exposed to 10nM of AlexaFluor 488 labeled-recombinant proteins in fresh serum-free media at 37°C. Proteins were incubated with B16 cells for 0.5 hour, with Colo857 for 1h, and with HCT116 p53^{+/+} and HCT116 p53^{-/-} for 2h. Thereafter, the incubation solutions were removed and the cells were washed three times with DPBS. Living cell preparations were observed with a LSM 710 confocal scanning laser microscope (Carl Zeiss, Jena, Germany), using a 63 \times , NA 1.2, C-apochromat water-immersion objective (Carl Zeiss). The experiment was carried out at 488 nm excitation and fluorescence was

collected with a 510-560 nm filter. 16 successive optical slices were captured along the cell z axis, with a step of 1 μ m.

3.6. Cellular uptake FACS analysis

To analyze the transduction efficiency and phosphatidyl serine (PS) exposure in response to recombinant protein treatment, a FACS analysis was performed. B16, Colo857, HCT116 p53^{+/+} and HCT116 p53^{-/-} cells (1 x 10⁵ cells/well) were cultured on 12-well plates until 70% of confluence. Cells were washed twice with DPBS and incubated with 10nM of AlexaFluor[®] 488 labeled-recombinant proteins in fresh serum-free culture medium for 2 hours at 37°C. As 488nm fluorescence positive control, recombinant proteins containing the MD₁₁ peptide fused to a non toxic eGFP reporter protein (MD₁₁-eGFP, 0.3 μ M) were incubated for 2 hours with cells. As toxicity control, doxorubicin (1 μ M) was incubated with cells 24 hours before analysis. After incubation, cells were trypsinized with 0.5% trypsin/EDTA solution (PAA, GE Healthcare) for 10 min at 37°C. The trypsin was then neutralized by adding complete medium, cells were washed twice with DPBS and then stained with Annexin V-APC and the vital dye 7-Amino-actinomycin (7-AAD, BD Biosciences). Cell-associated fluorescence was detected using a FACS Canto II BD Biosciences flow cytometer (equipped with 3 lasers). The 488nm and 633nm excitation and the 647nm emission filters (for 7AAD) and the 660/20 (for annexin V-APC) were used. The cytometer parameters were performed using the 7color set-up beads (BD) and the compensations were calculated with the Set-up compbeads kit from BD using antibodies for conjugates and cells for viability dye. The results are reported as the percent of fluorescent cells from living cells gate of 30000 events recorded and analyzed with the FACSDiva software.

4. Results

4.1. Expression and purification of recombinant fusion proteins

Cell-penetrating proteins are designed and developed as recombinant fusion proteins containing MD₁₁ cell-penetrating peptide attached to the N-terminus of eIF3f sequence [20] (Fig. 1A). We used the full length human protein (MD₁₁-wt eIF3f) and three mutants (Fig.

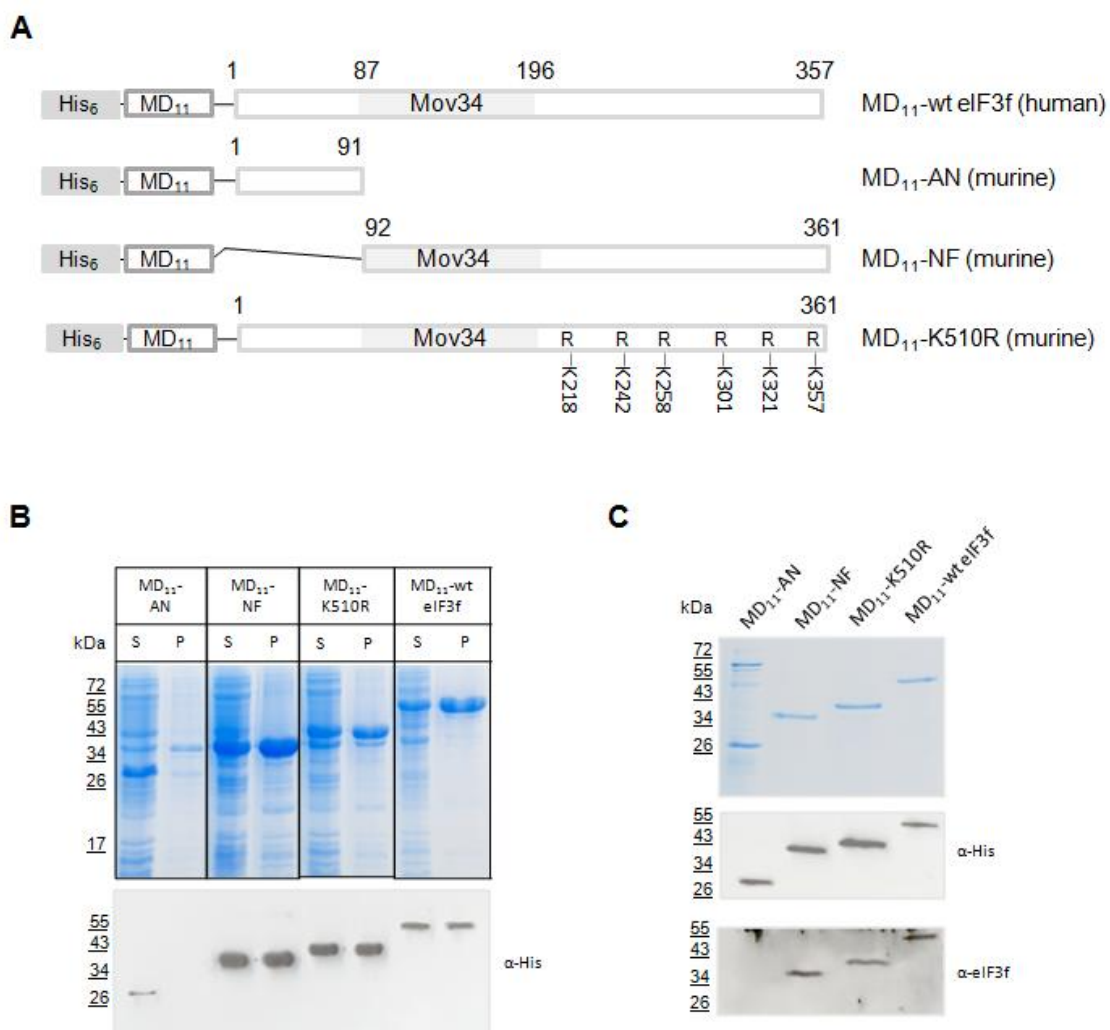


Figure 1. Design, expression and purification of MD₁₁-eIF3f recombinant fusion proteins. A) Scheme of recombinant MD₁₁-eIF3f constructs containing His tag for affinity purification. Mutations in which C-terminal lysines were replaced to arginines are indicated in MD₁₁-K510R mutant. B) Protein expression level in *E. coli*. After bacterial cell lysis, the soluble (S) and the insoluble (P) fractions were analyzed by SDS-PAGE, stained with Coomassie blue and identified by western blotting using an anti-His antibody. C) After purifications, soluble recombinant fusion proteins were analyzed by SDS-PAGE, stained with Coomassie blue and detected by western blotting with an anti-His antibody and an anti-eIF3f antibody.

1A): the N-terminal protein truncation lacking of Mov34 domain (MD₁₁-AN, residues 1-91), the C-terminal truncation containing the Mov34 domain (MD₁₁-NF, residues 92-362) and the ubiquitination-resistant mutant [19] in which six C-terminal lysines (from K5 to K10) are replaced by arginine residues (MD₁₁-K510R). The murine eIF3f amino acid sequence shows extensive homology with its human counterpart (92%), particularly in the region that contains the Mov34 domain.

The his-tagged recombinant fusion proteins were produced using an *E. coli* expression system (Fig. 1B) and purified by nickel affinity chromatography. With the exception of MD₁₁-AN, MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R were expressed in both soluble and insoluble fractions. The yields of purified soluble proteins were negligible when compared to the total production, presumably due to the lack of tag exposition in native structure. For this reason, MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R were purified from inclusion bodies under denaturing conditions and refolded by dialysis. The purity of the MD₁₁-eIF3f proteins was analyzed by Coomassie blue staining and by western-blotting using anti-His tag and anti-eIF3f antibodies (Fig. 1C). MD₁₁-AN was pure at around 75% and MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R at around 95%.

4.2. ⁵¹Cr release cytotoxicity assay

Cytotoxic activities of MD₁₁-wt eIF3f and MD₁₁-K510R were measured on different cell lines using a time course ⁵¹Cr release cytotoxicity assay (Fig. 2). As control of toxicity, cells were incubated with 1 μM doxorubicin which becomes toxic after 18h incubation. To discard any toxicity linked to buffer, cells were also incubated with protein-free buffer (50mM Na citrate pH 6.1, 50mM NaCl, 10% glycerol) in same volumes used for protein treatments. Recombinant MD₁₁-wt eIF3f and MD₁₁-K510R cytotoxic effects were screened on both tumor cells, such as human (Colo857) and mouse (B16) melanoma, human (LN229, T98G, U87) and mouse (GL26) glioblastoma, human osteosarcoma (Saos2), human cervix adenocarcinoma (HeLa), human breast adenocarcinoma (MCF7), Burkitt's lymphoma (Raji), human colorectal carcinoma (HCT116), and on immortal non-tumorigenic cell lines, such as mouse skeletal myoblasts (C2C12), human embryonic kidney cells (HEK293E), and human breast cells (MCF10A).

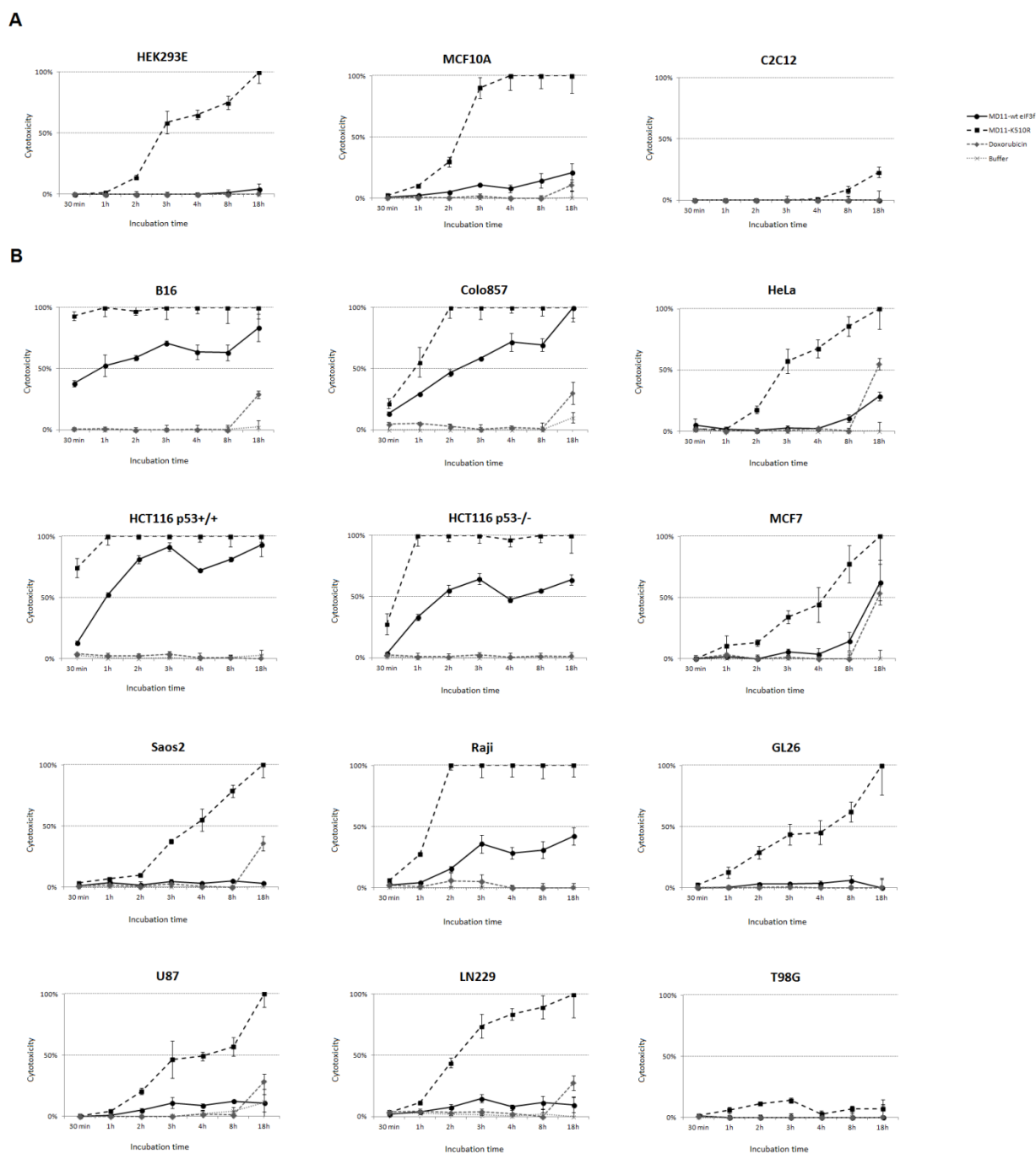


Figure 2. Screening of MD₁₁-wt eIF3f and MD₁₁-K510R cytotoxic effect by ⁵¹Cr-release assay on human and murine cell lines. 10nM of MD₁₁-wt eIF3f and MD₁₁-K510R were incubated with non-tumorigenic (A) and tumor cells (B) and ⁵¹Cr release measurements were performed 0.5; 1; 2; 3; 4; 8; 18 hours after protein incubation. Doxorubicin (1μM) was used as toxicity control and protein-free buffer was tested to discard any buffer-linked toxicity. Each data point is the mean of three values ± SD.

The cell-death values detected after MD₁₁-wt eIF3f and MD₁₁-K510R treatment are used as parameter to discriminate their cell-type dependency (Fig. 2). Generally, MD₁₁-K510R induced a stronger cytotoxic effect than wild-type eIF3f. As matter of facts 18 hours incubation with MD₁₁-K510R induced 100% death in HEK293E, MCF10A, MCF7, HeLa, Saos2, Raji and LN229, U87, GL26 cells, while at the same time point the wt protein mildly affect viability (from 0 to 50% in MCF7) (Fig. 2). Interestingly, T98G glioblastoma cells were resistant to both treatments (Fig. 2). C2C12 myoblast cell line viability is not affected by the treatment with recombinant eIF3f fusion proteins. This result is in line with previous observations demonstrating that eIF3f upregulation in muscle cells does not affect cell viability [17]. A massive and rapid cytotoxic effect was detected in melanoma (B16 and Colo857) and colorectal carcinoma (HCT116 p53^{+/+} and HCT116 p53^{-/-}) cells (Fig. 2). For instance, one hour treatment of MD₁₁-wt eIF3f induced 30% death in Colo857 and HCT116 p53^{-/-} and 50% in B16 and HCT116 p53^{+/+}, while MD₁₁-K510R killed as expected 50% of Colo857 cells and 100% of the others. In particular, the HCT116 cell model (p53^{+/+} and p53^{-/-}) was used to evaluate an eventual contribution of p53 in death induction. We found that at low dosages (2.5-10nM) HCT116 p53^{-/-} cells were more resistant to the protein treatment when compared to the wild type cell line. On the basis of these screening results B16, Colo857, HCT116 p53^{+/+} and HCT116 p53^{-/-} cells were chosen as target tumor models for following experiments.

4.3. Protein labeling and intracellular detection

To assess the ability of fusion recombinant proteins to transduce into cells, MD₁₁-AN, MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R were labeled with AlexaFluor488 dye and incubated with B16 cells for 30 minutes, with Colo857 for 1h, and with HCT116 p53^{+/+} and HCT116 p53^{-/-} for 2h. Treatment times were chosen according to ⁵¹Cr kinetics results (Fig. 2). The intracellular delivery of recombinant eIF3f was detected in living target cells by confocal laser scanning microscopy. Cellular Z-stack analysis (Fig. 3) revealed that all four recombinant proteins were detectable in cell cytoplasm, confirming that MD₁₁-bearing eIF3f proteins were able to penetrate cells. We also noticed that exogenous recombinant proteins were able to localize into cell nuclei, effect that has been already observed and reported for endogenous proteins [21, 22].

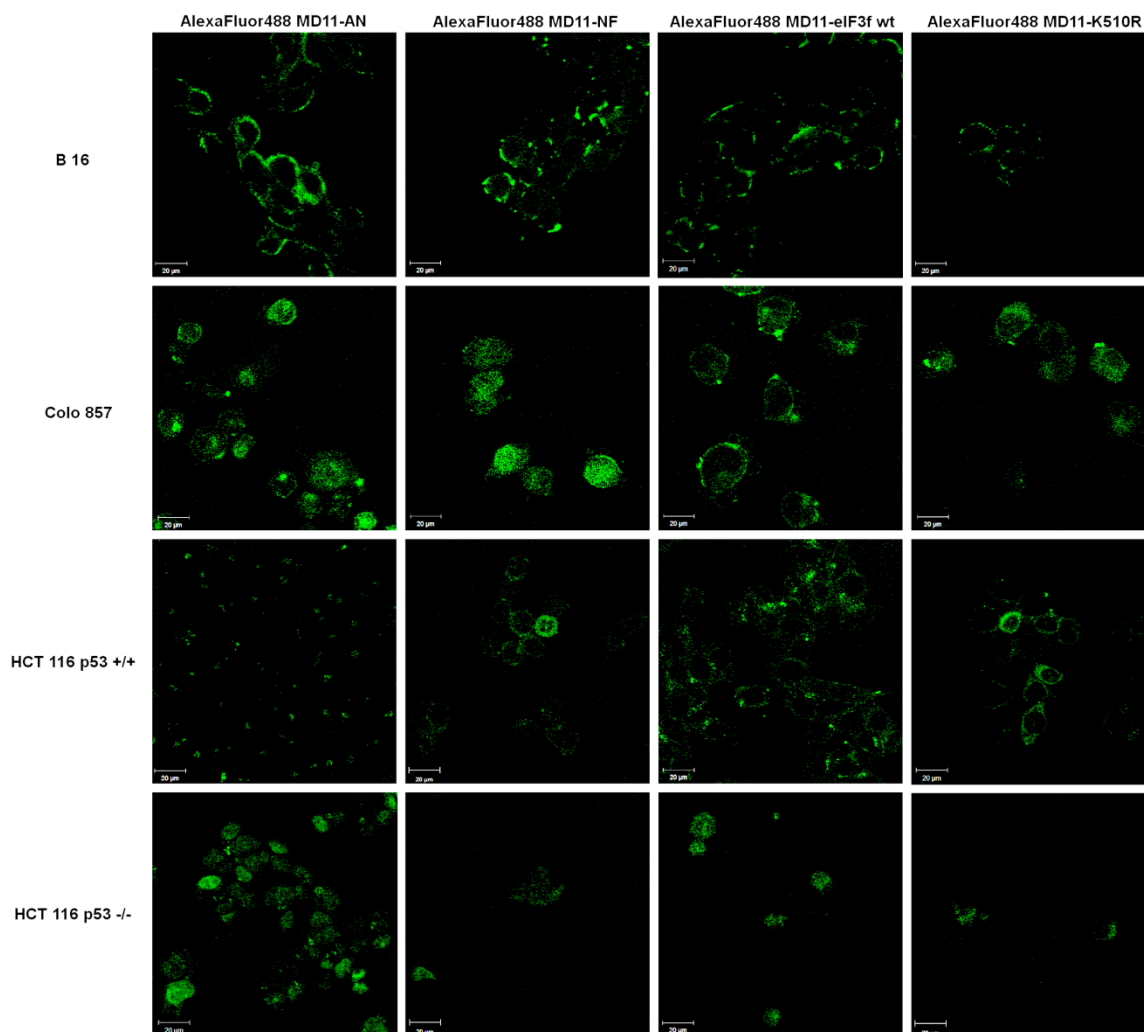


Figure 3. Confocal laser scanning microscopy images of living B16 (0.5h), Colo857 (1h), HCT116 p53 +/+ (2h) and HCT116 p53 -/- (2h) cells incubated with AlexaFluor 488-labeled MD₁₁-AN, MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R fusion protein (10nM). 16 successive optical slices were captured along the cellular z-axis with a step of 1 μm. The presented images correspond to the middle plan of cellular z-axis sectioning.

4.4. Cellular uptake and Annexin V FACS analysis

To estimate the transduction efficiency and the cell death induction, a FACS analysis using AlexaFluor488 labeled fusion proteins was performed (Fig. 4). Recombinant eIF3f proteins were efficiently transduced into selected cell lines (Fig. 4D). A simultaneous Annexin V-7AAD staining was used to quantitatively determine the percentage of cells undergoing apoptosis. Annexin V has a high affinity for membrane phospholipid phosphatidylserine (PS) which is translocated from the inner to the outer leaflet of the plasma membrane in apoptotic cells. 7-AAD is a viability probe that is excluded from viable cells and is internalized in dead cells. The FACS analysis (Fig. 4A) on B16, Colo857, HCT116 p53^{+/+} and HCT116 p53^{-/-} cells showed that the incubation with MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R increases the percentage of apoptosis compared to the negative controls (untreated cells, Fig. 4B). These results indicate that the cell death quantified by ⁵¹Cr release assay can be ascribed as apoptotic-like process linked to PS flip-flop. To exclude that the toxicity of fusion proteins was not induced by the MD₁₁ penetrating sequence, cells were also incubated with MD₁₁-eGFP reporter protein (at 30-fold higher dose than therapeutic proteins) and no effect on viability was detected (Fig. 4C). The effect of the four recombinant proteins was also investigated in normal BJ fibroblasts using Annexin V-7AAD staining (Fig. 8). Except for MD₁₁-K510R, the recombinant proteins do not affect the viability of normal cells, even after 8h treatment.

In addition, the apoptotic action is specific to full-length eIF3f or its C-terminal truncation, as demonstrated by the lack of significant death in cells incubated with MD₁₁-AN (Fig. 4A). These findings confirm that exogenous wt eIF3f, NF truncation or ubiquitination-resistant mutant induce apoptosis in melanoma and colorectal carcinoma cells.

4.5. ⁵¹Cr release assay on selected tumor cell lines

Once determined that the four recombinant proteins are internalized in target cells and that retain their therapeutic anti-cancer activity, we conducted a dose- and a time- course study using ⁵¹Cr release assay (Fig. 5). Cells were incubated with 2.5, 5, 10 and 50nM of each recombinant protein and measurements were taken at various times after protein incubation. As controls, cells were incubated with doxorubicin and with protein-free buffer (50mM Na

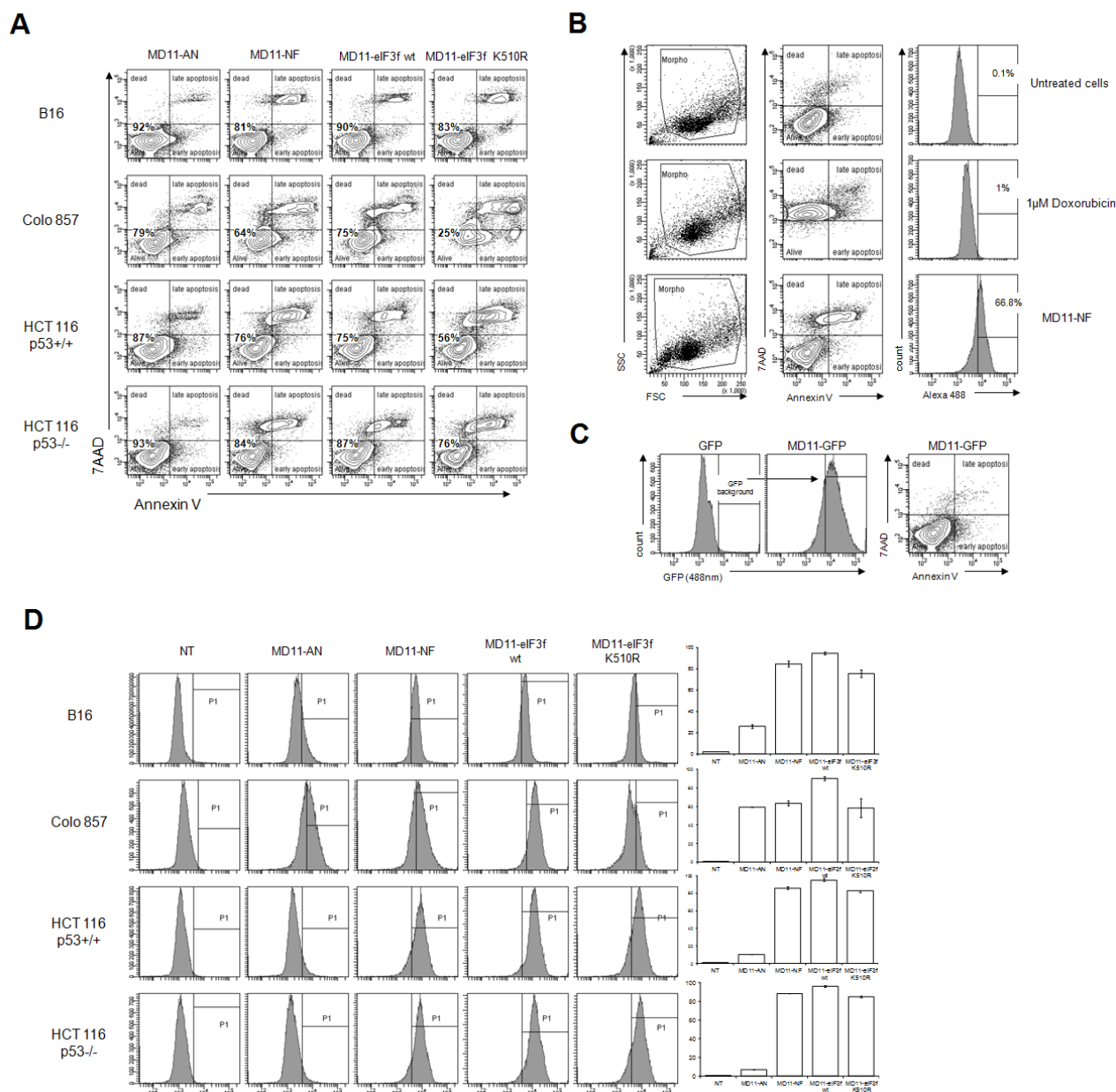


Figure 4. Annexin V-7AAD apoptosis assay and protein delivery efficiency analysis by flow cytometry. A) B16, Colo857, HCT116 p53 +/+ and HCT116 p53 -/- cells were incubated with AlexaFluor 488 labeled proteins (10nM) and stained with Annexin V-APC and 7-AAD. Alive cells show negative staining for Annexin V and 7-AAD. Cells undergoing apoptosis show positive staining for Annexin V and negative for 7-AAD, while cells positive for both Annexin V and 7-AAD are either in the end stage of apoptosis, undergoing necrosis or already dead. B) Examples of FACS profiles of untreated cells and doxorubicin-treated cells, used as controls for viability/toxicity setting gates. C) Representative plot of HCT116 p53 -/- cells incubated with 0.3 μ M MD11-eGFP, showing no toxic effect associated to the MD11-mediated protein internalization. D) Evaluation of delivery efficiency of AlexaFluor 488-labeled recombinant proteins in selected cells. Histograms represent percentages of internalized MD11-AN, MD11-NF, MD11- wt and MD11-K510R proteins in target cells.

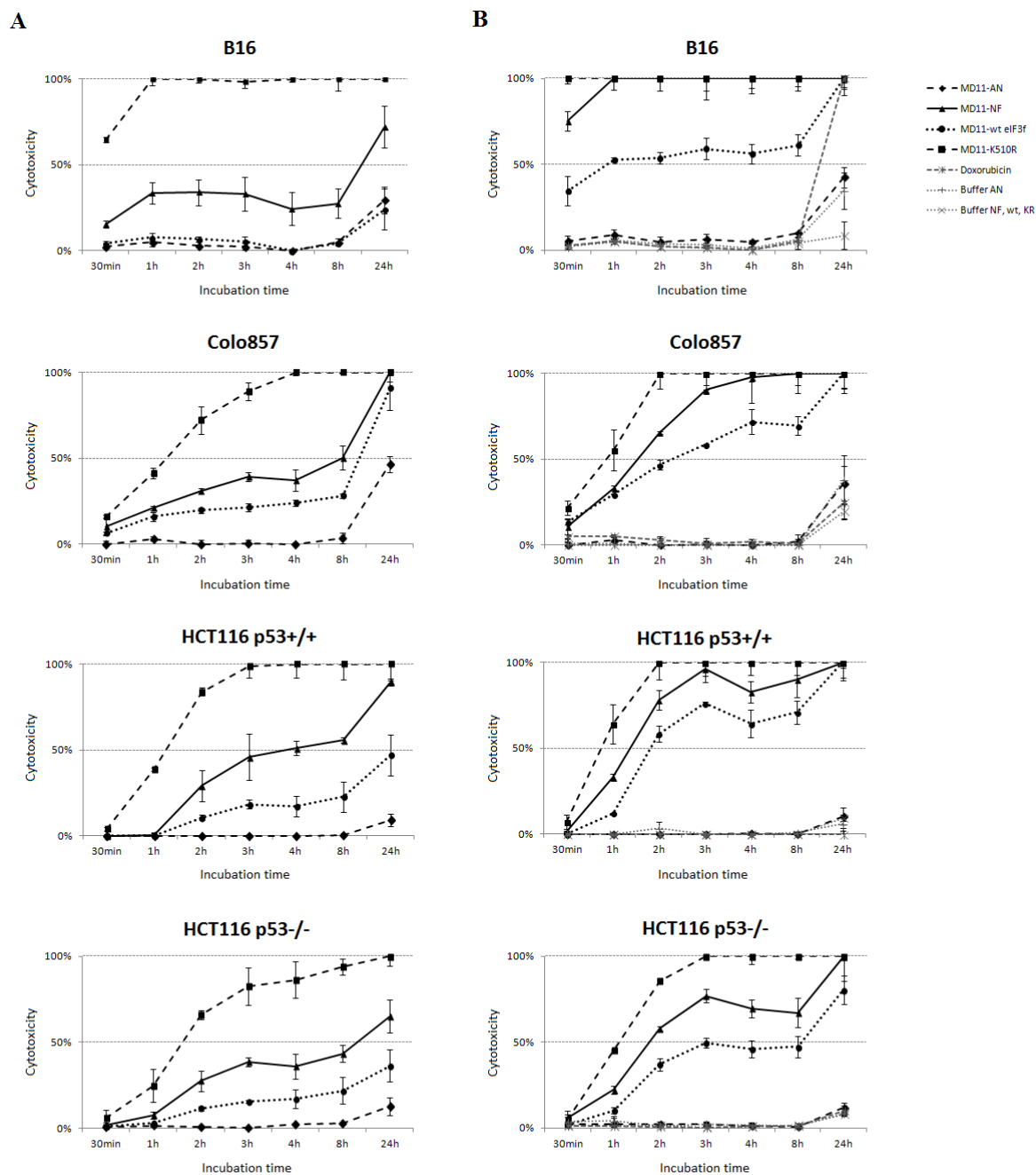


Figure 5. Time-dependent kinetics of MD₁₁-AN, MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R effect on B16, Colo857, HCT116 p53 +/+ and HCT116 p53 -/- cells. Treatment with 5 (A) and 10nM (B) of recombinant proteins were performed and ⁵¹Cr release measurements were collected 0.5; 1; 2; 3; 4; 8; 18 hours after protein incubations. Doxorubicin (1 μ M) was used as toxicity control and protein-free buffers were tested to discard any buffer-linked toxicity. Each data point is the mean of three values \pm SD.

citrate pH 6.1, 50mM NaCl, 10% glycerol for MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R and 25mM HEPES/KOH pH 7.0, 50mM NaCl and 10% glycerol for MD₁₁-AN) in same volumes used for protein treatments.

MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R induced inhibition of cellular proliferation in all target cell lines in a dose- and a time- dependent manner. At lower tested dose (2.5nM), the time required to obtain a maximum of 50% cellular death was 24 hours in both melanoma and colorectal carcinoma cell lines (Fig. 6). In murine melanoma cells, at 10nM concentration the 50% cellular death was achieved in 30 minutes after MD₁₁-K510R treatment and in 1 hour after MD₁₁-wt eIF3f treatment (Fig. 5B). At the same dose, in human melanoma cells (Colo857) and in colorectal carcinoma cells (HCT116 p53+/+) the time necessary for 50% inhibition of cellular proliferation was less than 1 hour for MD₁₁-K510R and 1.5 hours for MD₁₁-NF and 2 hours for MD₁₁-wt eIF3f (Fig. 5B). The same effect was reached in colorectal carcinoma cells lacking of p53 (HCT116 p53-/-) in about 1 hour for MD₁₁-K510R, 1.5h for MD₁₁-NF and 3 hours for MD₁₁-wt eIF3f, confirming that this cell line is more resistant than wild type to the death stimulus and that p53 may play a role in eIF3f-induced death (Fig. 5B). Apoptotic effects were detected also at lower doses (5nM) of recombinant proteins, but at longer times (24h, Fig. 5A). At 50nM doses, the apoptotic response curves reached the plateau within 30 minutes (Fig. 7). Treatments with the non-functional MD₁₁-AN did not affect cell viability when compared to control cells incubated with protein-free buffer (Fig. 5A,B).

5. Discussion

The f subunit is a negative regulator of translation initiation, and the decrease of its expression contributes to tumor cells' evading apoptosis via up-regulation of protein synthesis [12]. The endogenous level of eIF3f protein is downregulated in several cancers and in particular in melanoma and pancreatic cancer cells [10, 13, 14]. Advances in the understanding of the molecular mechanism behind cellular carcinogenesis have shown that ectopic expression of eIF3f by transient gene transfection inhibits translation, cellular growth and proliferation and induces apoptosis [10, 13, 14]. Increasing the intracellular level of eIF3f protein seems to be a crucial element for treating cancer. Due to the difficulties in developing safe and efficient gene-delivery methods and in controlling the levels of transient gene

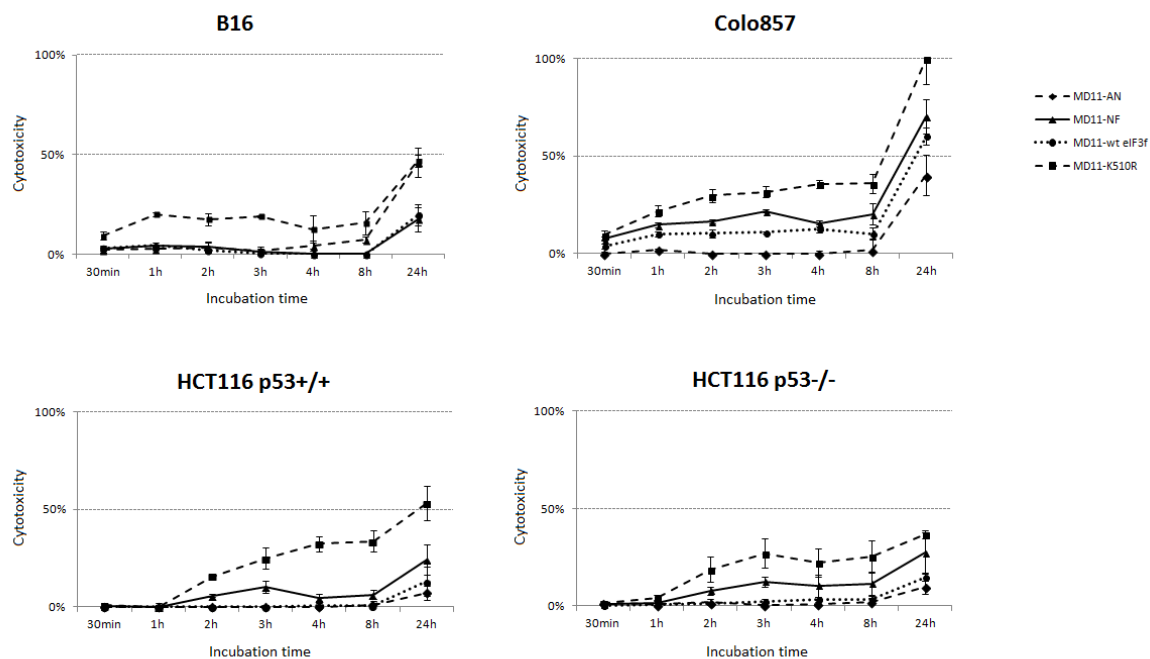


Figure 6. Time-dependent kinetics of MD₁₁-AN, MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R effect on B16, Colo857, HCT116 p53^{+/+} and HCT116 p53^{-/-} cells. Treatment with 2.5nM of each recombinant protein were performed and ⁵¹Cr release measurements were collected 0.5; 1; 2; 3; 4; 8; 18 hours after protein incubations.

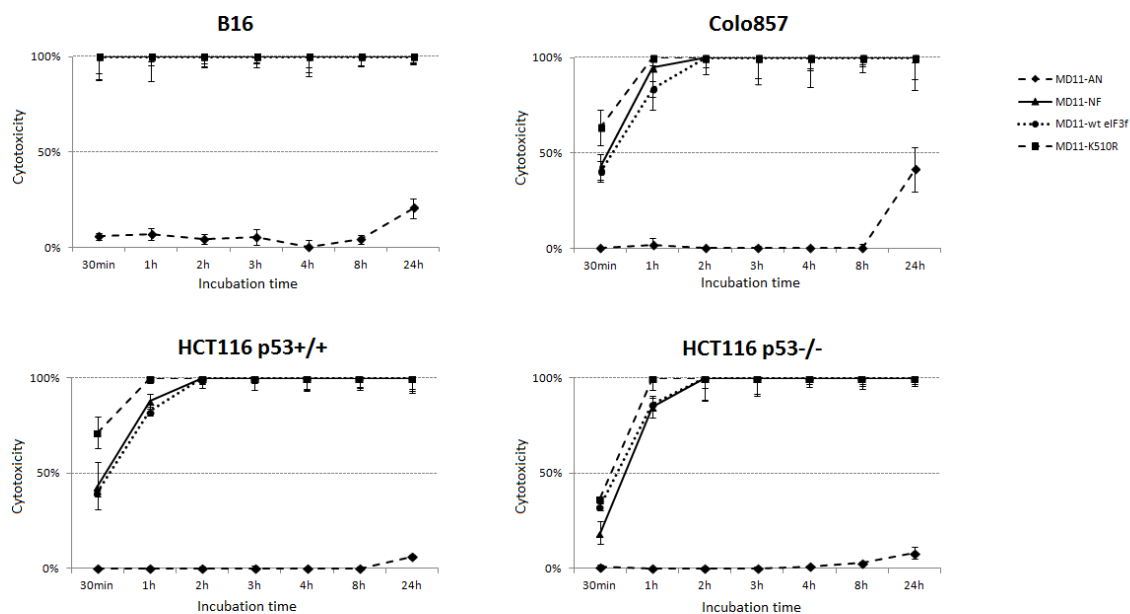


Figure 7. Time-dependent kinetics of MD₁₁-AN, MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R effect on B16, Colo857, HCT116 p53^{+/+} and HCT116 p53^{-/-} cells. Treatment with 50nM of each recombinant protein were performed and ⁵¹Cr release measurements were collected 0.5; 1; 2; 3; 4; 8; 18 hours after protein incubations.

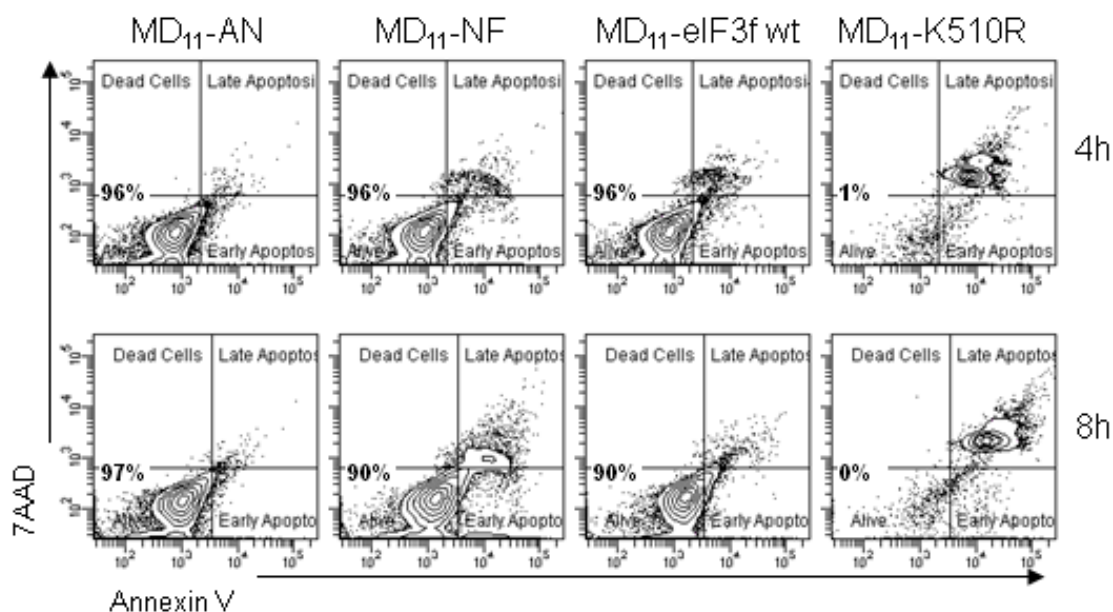


Figure 8. AnnexinV-7AAD apoptosis assay by flow cytometry. BJ human primary fibroblasts were treated for 4 and 8 hours with 10nM of MD₁₁-AN, MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R and stained with Annexin V-APC and 7-AAD. Alive cells show negative staining for Annexin V and 7-AAD. Cells undergoing apoptosis show positive staining for Annexin V and negative for 7-AAD.

expression, the protein therapy represents a valid alternative to gene therapy. To this aim, we developed an approach based on the direct delivery of functional exogenous eIF3f proteins into cells. Thus, the MD₁₁ cell-penetrating sequence [4] was fused to eIF3f therapeutic proteins, and the effect of their cellular internalization was monitored.

The first evidence on the efficient cellular uptake and consequent protein therapeutic effect arises from the results of ⁵¹Cr screening assay on different cell lines. MD₁₁ fused with wild-type eIF3f and its ubiquitination-resistant mutant (K510R) showed a cell-type dependent effect and, in the same cell line, they behaved differently. This difference confirms both the functional state and the expected intracellular processing of the delivered molecules. In particular, we observed a stronger MD₁₁-K510R-mediated death induction when compared to the wt molecules. Indeed, the C-terminal point mutations (K510R) prevent polyubiquitination of eIF3f and proteasome degradation, leading to increased *in cellulo* protein stabilization. Interestingly, in T98G and C2C12 cells any effect on the viability is observed. While the lacking of literature does not allow formulate any hypothesis on glioblastoma cells and eIF3f correlation, our result on C2C12 confirms that overexpression of eIF3f does not induce cell death but it acts as ‘cell growth enhancer’ instead of ‘death executor’ [17]. Among the tested cell lines, melanoma and colorectal carcinoma cells showed the most interesting death profiles and we decided to pursue our investigation on B16, Colo857, HCT116 p53^{+/+} and HCT116 p53^{-/-}.

Knowing that the Mov34 domain plays a crucial role in regulating the activity of eIF3f protein and its interaction with most identified partners [18], we engineered two eIF3f truncations: an N-terminal truncation (MD₁₁-AN) lacking of the Mov34 domain and a C-terminal truncation containing the Mov34 domain (MD₁₁-NF). Once incubated with target cells, MD₁₁-NF effectively suppresses target cancer cells proliferation with a higher efficiency than the wild type protein. In contrast, cells incubated with MD₁₁-AN show unchanged cell proliferation. These results demonstrate that the N-terminal part of eIF3f is not implicated in apoptosis-inducing activity, and that the Mov34 domain plays a key role in regulating the activity of this subunit in cancer cells.

When transduced by MD₁₁, fluorescently labeled wt, AN, NF and K510R eIF3f proteins localize into cytoplasm or nucleus of selected cell lines. Our findings are in line with previous

observations reporting that endogenous eIF3f can localize at both cytoplasm and nuclear levels [21]. During apoptosis, endogenous eIF3f is phosphorylated by CDK11 which is mainly a nuclear protein [21, 22] and can assemble with other eIF3 subunits to form nuclear complexes which are involved in functions other than translation initiation, such as ribosome biogenesis that takes place into nucleus [22]. The observation of nuclear localization together with the apoptotic effect observed by Annexin V assay strongly suggests that the exogenous and endogenous eIF3f are subjected to similar intracellular processing. It has been shown that in melanoma and pancreatic cancer cells eIF3f can induce apoptosis in caspase 3/7 and in a Bcl-2, Bax or Bcl-XL- independent manner [10, 13, 14].

An ideal protein-derived therapeutic drug should ideally be able to trigger apoptosis in cancer cells without inducing side effects in normal cells. Analyzing the results obtained with the recombinant eIF3f proteins, we can consider that MD₁₁-NF, MD₁₁-wt eIF3f and MD₁₁-K510R possess an interesting therapeutic potential for tumor treatment. As matter of fact MD₁₁-K510R shows a high toxic effect on both normal and tumor cells, while MD₁₁-wt eIF3f has lesser toxicity on tumor cells and no effect on normal cell viability.

Considering this MD₁₁-wt eIF3f peculiar property, we can claim that this molecule is a possible and suitable candidate for melanoma and human colorectal carcinoma treatment. Some additional investigations need to be conducted for deciphering the detailed molecular mechanisms behind eIF3f action and its effects on cell viability before validating its therapeutic efficiency in *in vivo* models. Optimization of this protein-derived drug, including the fusion to tumor homing peptide sequences, will also be considered.

In conclusion, the work presented here establishes the first proof of concept on the use of MD₁₁-based delivery system as an efficient tool for transporting functional protein cargos in living cells, and for developing protein therapy strategies for melanoma or colorectal carcinoma. This technology opens a broad range of possible applications; in fact the controlled intracellular protein delivery can be exploited for treating other malignancies or diseases by fusing the MD₁₁ peptide sequence to other therapeutic target proteins.

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CHAPTER III

Investigation of MD₁₁ cell-penetrating properties in yeast cells

Roberta Marchione, David Daydé, Jean-Luc Lenormand, Muriel Cornet, ZEBRA cell-penetrating peptide as an efficient delivery system in *Candida albicans*, *Biotechnology Journal*; 2014, Jan 22. [Epub ahead of print]; PMID: 24449606; doi: 10.1002/biot.201300505.

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Preface

The action of CPPs has been extensively studied in various mammalian cells, but only few reports describe their ability to traverse the cell wall of yeasts. Yeast cells share most of the structural and functional features of higher eukaryotes. Their rapid growth and ease of genetic manipulation make them an ideal model for eukaryotic cell biology and for many genetic studies.

Yeast cell membranes are coated with an extracellular matrix that provides a largely inert structural framework and protective barrier. The yeast cell wall is schematically organized into a common central layer composed of a branched β -1,3-glucan cross-linked to chitin and an outer layer, which composition varies with the morphotype, the growth stage and the fungal species (Latge, 2010). Because of its thickness and rigidity, the cell wall has been considered for many years as an impenetrable surface.

Some CPPs, such as penetratin (Holm *et al.*, 2005), pVEC (Holm *et al.*, 2005), TP10 (Nekhotiaeva *et al.*, 2004), and Tat (Jung *et al.*, 2006) enter yeast cells when fused to the reporter protein GFP or when labeled with fluorescent dyes. Even though their entry is non-toxic for mammalian cells, peptide uptake in yeasts is often associated to cell leakage and death (Garibotto *et al.*, 2011; Jung *et al.*, 2006; Masman *et al.*, 2009; Nekhotiaeva *et al.*, 2004). As consequence, these CPPs are used mainly as antimicrobial peptides (AMPs) or antifungal agents. The distinct effects reported for CPPs are probably linked to the different composition between mammalian and yeast membranes (Henriques *et al.*, 2006).

This chapter explores the MD₁₁ penetration ability into yeasts, and in particular provides the proof of concept that this peptide penetrates into *Candida albicans* cells. *C. albicans* is a commensal yeast, meaning that it is a ubiquitous component of the normal human microflora. However, in immunocompromised individuals it may become invasive and cause a condition called candidiasis. Contrary to other known CPPs, MD₁₁ efficiently mediates the internalization of eGFP cargo proteins into *C. albicans* cells with any associated toxic effect. In the following paper, the MD cell-penetrating peptide used for yeast uptake experiments corresponds to MD₁₁ sequence.

These results open up various possibilities for functional research applications. Indeed, studies with yeasts have yielded basic insights into cellular processes applicable to all eukaryotes, such as mitochondrial genetics, membrane biogenesis and gene silencing (Brambl, 2009). Thus, a possible application of MD₁₁ could be its use as carrier for macromolecules which monitor or modify specific intracellular processes, providing an efficient way to study the temporal and the spatial regulation of cellular basic functions. Furthermore, considering the pathogenicity of *C. albicans*, some therapeutic applications could be also conceived, for example by using MD₁₁ peptide to deliver cytotoxic agents or specific inhibitors of yeast cells.

ZEBRA cell-penetrating peptide as an efficient delivery system in *Candida albicans*

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Abbreviations: CPP, cell-penetrating peptide; GFP, green fluorescent protein; eGFP, enhanced green fluorescent protein; MD, minimal domain; IPTG, isopropyl β -D-1-thiogalactopyranoside ; DTT, dithiothreitol; OD, optical density; BCA, bicinchoninic acid; PBS, phosphate buffered saline; YPD, yeast extract peptone dextrose; FACS, Fluorescence-activated cell sorting; CFU, colony forming unit.

1. Abstract

There is increasing interest in drug delivery systems, such as nanoparticles, liposomes and cell-penetrating peptides, for the development of new antimicrobial treatments. In this study, we investigated the transduction capacity of a carrier peptide derived from the Epstein-Barr virus ZEBRA protein in the pathogenic fungus *Candida albicans*. ZEBRA-Minimal domain (MD) was able to cross the cell wall and membrane, delivering eGFP to the cytoplasm. Uptake into up to 70% of the cells was observed within two hours, without toxicity. This new delivery system could be used in *Candida albicans* as a carrier for different biological molecules including peptides, proteins and nucleic acids. Thereby, in antifungal therapy, MD may carry promising bioactive fungal inhibitors that otherwise penetrate poorly into the cells. Furthermore, it will be of interest for deciphering molecular pathways involving cell-cycle control in yeast or signalling pathways. Short interfering peptides could be internalized by this system, providing new tools for the inhibition of metabolic or signalling cascades essential for the growth and virulence of *C. albicans*, such as yeast-to-hyphae transition, cell wall remodelling, stress signalling and antifungal resistance. These findings create new possibilities for the internalization of cargo molecules with applications for both treatment and functional analyses.

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CONCLUDING REMARKS AND PERSPECTIVES

In this thesis it is shown that the MD₁₁ cell-penetrating peptide deriving from the EBV ZEBRA protein can be employed as protein carrier in mammalian and yeast cells. In the first chapter, its endocytosis-independent mechanism of internalization is elucidated, opening the perspectives for future therapeutic applications. This uptake mechanism is determined by hydrophobic amino acids located in the peptide C-terminal end, which contribute to internalization efficiency and regulate the MD₁₁ endosomal escape.

MD₁₁-mediated delivery of functional proteins has been explored in Chapter II with the aim to develop a novel therapeutic approach for cancer treatment. The used strategy is based on the replenishment of the intracellular levels of eIF3f factor (downregulated in many cancers), with the consequent activation of apoptosis in tumor cells. To enhance protein stability and extend its therapeutic effect *in cellulo*, an ubiquitination-resistant version has been engineered. Results discussed in this chapter demonstrate that treatment with these recombinant proteins significantly reduces *in vitro* tumor viability. Therefore, MD₁₁ can be used as protein therapy “drug” because eIF3f-derived molecules are delivered intracellularly in an active form and their biological effect is detectable in few minutes after internalization. These findings confirm the potential of MD₁₁-based transfer technology and open up other therapeutic perspectives.

While the MD₁₁ ability to enter into different cell types represents a great advantage for various applications, its lack of cell specificity creates a significant challenge for the development of targeted molecular therapies. In our specific case, this drawback has been circumvented by delivering a protein showing a cell-type specific function (MD₁₁-wt eIF3f). For eIF3f ubiquitination-resistant mutant as well as for other MD₁₁-based protein therapeutic applications some improvements, combining both targeting and internalization advantages, should be exploited. Cancer cells are very often targeted for therapeutic purposes as their metabolism is different from that of normal cells. To improve the MD₁₁ delivery specificity, different strategies could be used such as the coupling with ‘tumor-homing peptides’ or with activatable peptides, which are cleaved in acidic microenvironments or by specific matrix metalloproteinases.

Some points remain to be investigated and optimized. For instance, it would be interesting to explore the molecular mechanisms responsible for the MD₁₁-eIF3f cell-type

specificity and clarify whether this effect is induced by the interaction with other intracellular partners (that are differently expressed between normal and tumor cells) or it is dependent from post-translational modifications that can elicit specific signaling pathways. In addition, it would be worthwhile to characterize the exact cell death molecular mechanism *in vitro*. Finally, to fully validate the tumor-suppressor effectiveness of MD₁₁-eIF3f proteins, *in vivo* studies are required. The selection of topical accessible tumors such as melanoma could represent the strategy of election to avoid side effects. Indeed, the direct delivery of recombinant proteins on targeted cells or in their close vicinity could be a means to prevent their *in vivo* spreading. Furthermore, these investigations should be combined with *in vivo* analysis of peptide biodistribution.

For the purpose of this thesis, only the apoptosis-inducing activity of eIF3f has been investigated. As described above, eIF3f protein plays a role of ‘growth enhancer’ in muscle cells. For this reason, the therapeutic efficiency of MD₁₁-eIF3f recombinant proteins should be tested in this cell type in order to inhibit atrophy and prevent its progression into irreversible cachexia.

The proof of concept that MD₁₁ can penetrate in yeast cells is provided in the last chapter. Although this study is still in its infancy, it holds some promising and exciting applications (previously discussed) both in terms of basic insights into cellular processes and therapeutic applications.

In summary, even though the MD₁₁ potential as protein delivery system is evident, some improvements in fusion protein formulation and *in vivo* studies are still necessary to completely validate and strengthen the effectiveness of its therapeutic applications.

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