TITLE PAGE

CELL DIVISION RESPONSIVE PEPTIDES FOR OPTIMIZED PLASMID DNA DELIVERY: THE MITOTIC WINDOW OF OPPORTUNITY?

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Running head: cell division responsive peptides

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ABSTRACT

The delivery of plasmid DNA remains hard to achieve, especially due to the presence of the nuclear membrane barrier. During cell division, however, the nuclear membrane is temporarily disassembled. We evaluated two different strategies to optimize plasmid DNA delivery in dividing cells: 1) phosphorylation responsive peptides that release plasmid DNA preferentially during mitosis and 2) chromatin targeting peptides to anchor plasmid DNA in newly formed nuclei upon cell division. Peptide/DNA particles alone were not efficient in penetrating cells. Upon co-delivery with lipid-based carriers, however, transfection efficiency drastically improved when compared to controls. For the phosphorylation responsive peptides, the presence of the phosphorylation sequence slightly increased transfection efficiency. For the chromatin targeting peptides, however, the chromatin targeting sequence did not seem to be the main reason for the improvement of transfection efficiency when applied in living cells. In conclusion, the pre-condensation of plasmid DNA with peptides improves lipid based delivery, but the nature of the peptides (cell responsive or not) does not seem to be the main reason for the improvement. It seems that the nuclear entry of foreign plasmid DNA is still under tight control, even during the mitotic window of opportunity.

keywords: chromatin targeting, plasmid DNA, cell division, phosphorylation responsive gene delivery, ternary complexes.

INTRODUCTION

The delivery of plasmid DNA remains an attractive but challenging goal in non-viral gene therapy. While some viruses have evolved to make use of the cellular machinery in translocating their genome to the host nucleus, non-viral gene delivery particles still struggle to bring their pDNA through the nuclear membrane barrier. In non-dividing cells, pDNA should gain access to the nucleus via the nuclear pore complexes (NPCs). This tightly regulated association of nucleoporins contains a central channel of +- 9 nm width through which translocation of cargo from the cytoplasmic sight to the nucleoplasm should take place. Upon the presence of so-called nuclear localization signals (NLS), the central channel can widen up to 40 nm, increasing the permeability of the NPCs to a certain extent [1]. Several strategies to increase the translocation of pDNA through NPCs have been evaluated, such as targeting with NLSs or dilation of the NPCs with corticosteroids [2-6]. Although some of them have proven useful, the efficiency to cross the nuclear membrane of non-dividing cells remains low.

It is generally accepted that cell division increases the transfection efficiency of pDNA. During cell division, the nuclear membrane is temporarily disassembled, leading to the 'mitotic window of opportunity' in which the probability of pDNA to reach the nuclear interior of the newly formed daughter cells apparently increases significantly [7, 8]. In a recent review, we proposed several strategies to enhance pDNA delivery during mitosis [9]. One of these strategies is to anchor pDNA to the chromatin during cell division, so that the nanoparticles efficiently are enclosed in the nuclei of the daughter cells. This strategy is in fact used by some retroviruses and some latent DNA viruses taking advantage of mitosis [10-13]. Binding of DNA or DNA nanoparticles to chromatin could both enhance the nuclear inclusion and increase the probability that pDNA is passed on during subsequent cell divisions. Using the Xenopus egg extract assay, we already evaluated the chromatin targeting properties of AT hooks derived from the endogenous Mel-28 and HMGA2β [14]. Chromatin targeting was improved 3-fold, but did not translate into an increase of transfection efficiency inside living cells.

In this study, we aimed to evaluate two additional strategies to increase pDNA delivery during mitosis. In the first strategy (Fig 1), we use peptides containing the consensus recognition sequence S/TPXK/R from Cyclin dependent Kinase 1 (CdK1). Cdk1 is mainly active during the different stages of mitosis such as nuclear envelope breakdown, spindle formation and anchoring of chromosomes to the spindle [15]. At the onset of mitosis, CdK1 is expected to phosphorylate the recognition sequence of the pDNA/peptide complexes. The introduction of negative charges could then decrease the peptides affinity to pDNA and promote the release of pDNA from the complexes. We hypothesize that the efficiency of pDNA delivery in dividing cells is strongly determined by the amount of intact pDNA present at the moment of cell division. This amount is dictated by two phenomena: i) the influx of pDNA in the cytoplasm resulting from cellular uptake, endosomal escape and release from the carrier and ii) the efflux of pDNA determined by degradation and/or immobilization due to binding to intracellular compartments. Therefore, developing pDNA carriers that would preferentially release the pDNA at the moment of cell division could be beneficial, by limiting the time frame in which cytoplasmic degradation of the nucleic acids can take place.

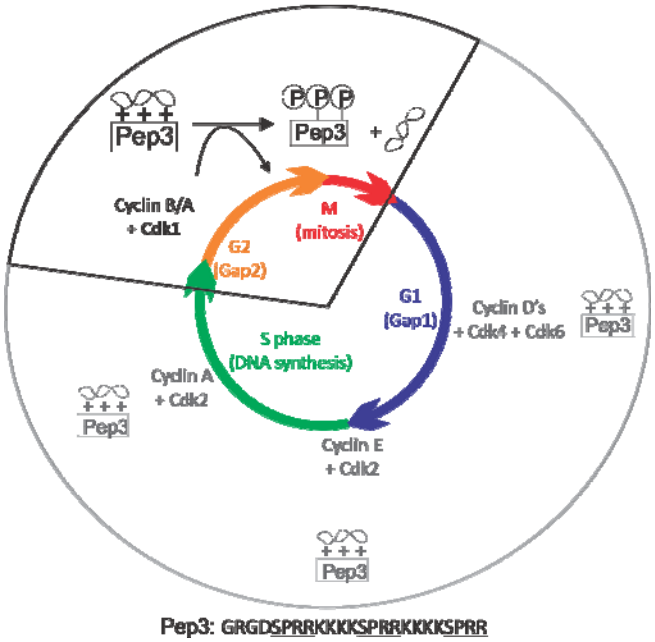


Fig. 1. Strategy 1: phosphorylation responsible peptides (pep3) for specific release of pDNA at the onset of cell division (M: mitosis). During Gap 1 (G1), DNA synthesis (S phase) and Gap 2 (G2 phase), pep3 is not phosphorylated and is expected to stay in complex with plasmid DNA. At the onset of cell division, Pep3 is phosphorylated by Cdk1 at the three recognition sites, leading to less affinity to pDNA and release of pDNA at the beginning of M phase.

In a second strategy, we aimed to anchor pDNA/peptide nanoparticles to chromatin by the use of peptides containing AT-hook sequences, or the consensus sequence from herpes virus LANA (latency-associated nuclear antigen) (Fig 2). LANA is responsible for attaching viral genomes to the histone H2A-H2B dimer at the surface of mitotic chromosomes [10, 16], while AT-hooks (consensus motif PRGRP) are DNA binding motifs that bind AT-rich regions on metaphase chromosomes [17]. The chromatin binding motifs were incorporated in synthetic peptides equipped with additional lysines, to create polycations with sufficient DNA condensing properties. As depicted in Figure 2, chromatin targeted particles could bind to the chromatin during cell division and subsequently be included in the newly formed nuclei of the daughter cells.

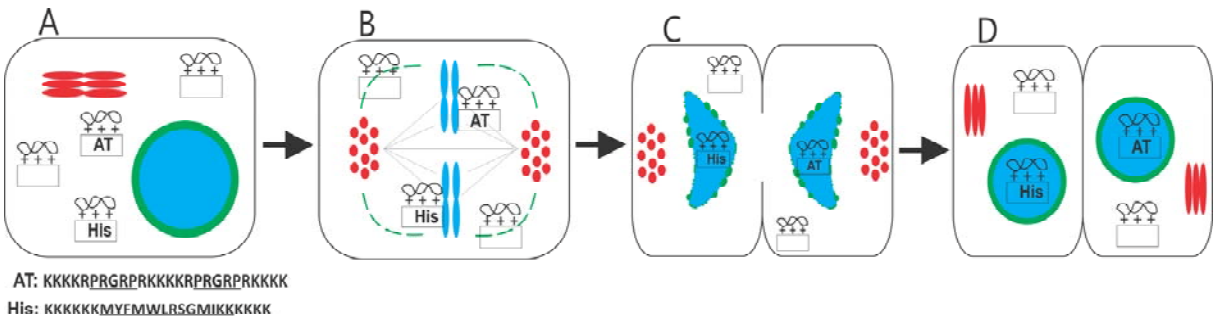


Fig 2. Strategy 2: peptides with chromatin targeting properties (AT and His) are used to bind plasmid DNA (A). During cell division, the nuclear envelop (green) is temporarily disassembled and the complexes can anchor to the chromatin (blue) (B). When the daughter nuclei are formed, targeted complexes are entrapped in the nuclear interior, while non-targeted complexes are not (C-D). Red: golgi apparatus.

These synthetic peptides could prove interesting candidates for non-viral gene delivery. To clearly test whether the observed effects are due to the cell responsive character of the peptides, however, the use of the right controls is crucial [18]. For the chromatin targeting strategy, oligolysines (repeated lysines) were used as a non-targeted control peptide. For the phosphorylation responsive peptides, the serine was replaced by a glycine residue, making them inert for the Cdk1 activity. To maximally investigate the role of cell division, transfection efficiencies of the synthetic peptides were evaluated in asynchronously dividing, synchronously dividing and arrested (non-dividing) cells.

MATERIALS AND METHODS

Materials

As phosphorylation sequence, SPRR was chosen based on the CdK1 phosphorylation consensus sequence S/TPXK/R. Peptides with 1 (Pep1: Ac-GRGDSPRR), 2 (Pep2: Ac-GRGDSPRRSPRR) or 3 (Pep3: Ac-GRGDSPRRKKKKSPRRKKKKSPRR) phosphorylation sites were ordered as well as a control lacking the phosphorylation site (Pep3mut: Ac-GRGDGPRRKKKKGPRRKKKKGPRR).

As chromatin binding peptides, an AT-hook was ordered based on the consensus sequence PRGRP, with additional lysines to create a polycation for pDNA binding (AT: Ac-KKKKRPRGRPRKKKKRPRGRPRKKKK). As histone-binding protein, the motif MYFMWLRSGMIKK known to bind to H2A-H2B dimers in mitotic chromosomes was flanked with extra lysine residues to provide the peptide with extra positive charges for pDNA binding (His: Ac-KKKKKKMYFMWLRSGMIKKKKKK). All peptides were ordered in acetylated form (Ac-) from Caslo Laboratory (Lyngby, Denmark). As a control, oligo-L-lysine (OLL, 20 lysines) and oligo-D-lysine (ODL, 10 lysines) were ordered from Sigma (Diegem, Belgium).

Commercial available Lipofectamine®, Oligofectamine® and RNAimax® were from Invitrogen (Merelbeke, Belgium).

pDNA production & labeling

Heat competent *E. coli* transformed with the gWIZ-GFP plasmid DNA (GeneTherapy Systems®, San Diego, California) were grown in LB medium with kanamycin at 37 °C for 20 h. pDNA was isolated and purified with the Qiafilter Plasmid Giga Kit (Qiagen®, Venlo, The Netherlands) according to the manufacturer's instructions. For fluorescence microscopy the pDNA was labelled with Cy5 according to the manufacturer instructions (Label IT kit, Mirus Bio Corporation, WI, USA).

Complex formation

Complex formation was performed by mixing equal volumes of 0.2 µg/µl pDNA with peptide solutions with a concentration determined by the wanted N/P ratio. To calculate the N/P ratio, we considered the fact that 1 µg/µl of pDNA contains 3 mM of negative charges coming from phosphate (mol P). The number of protonatable nitrogens per mol peptides was calculated by counting the number of basic amino acids lysine (K) and Arginine (R) and the end standing primary amine (mol N). Peptide stock solutions containing 60 mM of protonatable nitrogens were prepared for each peptide. Complexes were prepared in 20 mM HEPES buffer, with N/P ratio's ranging from 0 to 20, vortexed and left to equilibrate for 30 minutes. For preparing ternary complexes, plasmid/peptide complexes were diluted in optiMEM containing RNAimax® in a ratio 1:0.5; 1:1; 1:2 or 1:3 (µg pDNA:µl carrier). For example complexes prepared from 15 µl 0.2 µg/µl pDNA (containing 3 µg of pDNA in total) were diluted in optiMEM containing 1.5, 3, 4 or 6 µl of RNAimax®.

Complex characterization

To follow complexation in function of N/P ratio, pDNA/peptide complexes were loaded on 1% agarose gel at a concentration of 0.2 µg pDNA per well and left to run for 30 minutes at 100V. Afterwards, the position of the pDNA was determined by ethidiumbromide staining followed by UV illumination. The hydrodynamic size and zeta potential of the complexes was checked by respectively dynamic light scattering and surface potential measurements (Zetasizer 2000, Malvern, Worcestershire, UK) in 20 mM Hepes buffer.

Cell culture

HeLa cells and A549 cells were maintained at 37°C (5% CO_2) in Dulbecco's modified Eagle's medium supplemented with growth factor F12 (DMEM:F12 (1:1)), 10% heat inactivated fetal bovine serum, 2 mM L-glutamine and 100 µg/ml penicillin/streptomycin. All cell culture reagents were purchased from GibcoBRL (Merelbeke, Belgium). Cells were plated 1 day prior transfection in 12 well plates at

125.000 cells/well. Ternary complexes were prepared as described above and incubated with the cells in optiMEM for 4 h. For transfection experiments, cells were washed with PBS and fresh culture medium was added. Flow cytometry with a BD FACSCalibur (BD, Erembodegem, Belgium) was used to determine the percentage of GFP-positive cells and the fluorescence intensities 24h after transfection.

For cellular uptake, cells were incubated with complexes containing red labelled pDNA. After 4 hours, cells were trypsinized and suspended in flow buffer (1% bovine serum albumin in PBS). The red fluorescence from the plasmids was measured by flow cytometry. Alternatively, the cells were analyzed after 4 h by confocal laser scanning microscopy (Nikon EZC1-si, Nikon Instruments Europe B.V., Brussels, Belgium).

Cell synchronization (double thymidine block)

For the experiments on non-synchronized, synchronized and arrested cells, three sets of 12-well plates were prepared. The cells were seeded at 50.000 cells per well. Non-synchronized cells were left to grow in medium without thymidine until the moment of transfection. For the synchronization and arrestation, the culture medium was replaced one day after seeding with culture medium containing 2 mM thymidine (Sigma-Aldrich, Bornem, Belgium) during 19 hours. Then, the cells were washed and incubated with fresh medium without thymidine. After 9 hours, 2 mM thymidine was added to the culture medium. After another 17 h of incubation, cells were washed and incubated with fresh medium without thymidine to obtain a synchronized cell culture. To keep cells in the arrested (non-dividing) state, cells were further incubated with medium containing 2 mM thymidine during the transfection. Synchronized cells were transfected in G2 phase, about 6-7 hours before cell division was expected to occur. Cell extract from synchronized cells in G2/M phase and non-synchronized cells was prepared using the ProteoJET^{TM} Cytoplasmic and Nuclear protein extraction kit, according to the manufacturers instructions (Fermentas Life Sciences, Germany). Prior to extraction, a protease inhibitor cocktail (Complete Mini, EDTA free, Roche Diagnostics, Mannheim,

Germany) was added to the supplied cell lysis buffer. Cell lysate was stored at -70°C. To test the phosphorylation ability and subsequent pDNA release, pep3/pDNA complexes at N/P ratio 5, 10 and 20 were incubated in buffer, cell extract from non-synchronized and synchronized cells during 2 hours at 37°C. Subsequently, the complexes were loaded on an agarosegel as described above.

Electroporation

HeLa cells were electroporated at 1.2 x 10^6 cells/400 µl optiMEM containing the pDNA/peptide complexes at 260 V and 850 µF. Then, cells were centrifuged and washed with PBS to remove remaining complexes from the culture medium. Cells resuspended in culture medium were seeded in 12 well plates and left to incubate for 24 hours before assessing the GFP positive cells by flow cytometry as mentioned above. For uptake experiments, the amount of red labelled pDNA/peptide complexes that reached the cytoplasm was assessed immediately after electroporation.

Xenopus nuclear envelope reassembly (XNER) assay

Sperm chromatin was prepared from *Xenopus leavis* sperm (*Xenopus laevis* testis were kindly provided by Kris Vleminckx Lab, VIB, Ghent) as previously described by Hetzer *et al.* (2000) [19]. Cytosolic extract and membrane fraction needed for the XNER assay were isolated from *Xenopus laevis* eggs. To study the nuclear inclusion in the XNER assay, 1 µl of Cy5-pDNA/peptide nanoparticles were added to a mixture of sperm chromatin (0.6 µl) and cytosolic extract (20 µl) and incubated in a water bath at 20 °C for 20 minutes. Then, 1 µl membrane fraction, 1 µl energy mix (127.5 mg/ml creatin phosphate, 2.5 mg/ml creatin kinase, 25 mM ATP and 25 mM GTP) and 1 µl glycogen (200 mg/ml) were added and this mixture was incubated in a water bath at 20 °C to allow the formation of artificial nuclei. After 90 minutes the artificial nuclei were stained with $DiIC_{18}$ (membrane staining) and DAPI (chromatin staining) and the enclosure of the nanoparticles in the nuclei was visualised with fluorescence confocal laser scanning microscopy (CLSM) with a Nikon EZC1-si.

RESULTS & DISCUSSION

Characterization of phosphorylation responsive pDNA/peptide complexes

Peptides containing 1, 2 or 3 phosphorylation sequences were used to complex plasmid DNA. Figure 3 shows the gel retardation assay, demonstrating that both pep 1 (containing 4 mol positive charges per mol peptide) and pep2 (containing 6 mol positive charges per mol peptide) were not sufficient to condense pDNA in nanoparticles that are retained in the wells of the gel. Even up to N/P ratio 20, free pDNA is observed to migrate into the agarose gel. The peptides pep3 and pep3 mut, which each contain 16 mol positive charges per mol peptide, did succeed in forming complexes with the pDNA starting from N/P ratio 2.5. It should be noted that the ethidiumbromide staining in the wells of the gels becomes less intense with increasing N/P ratios. This points out that the pDNA becomes less accessible to this intercalating dye, most likely related to a stronger compaction of the pDNA with higher N/P ratios. The size and zeta potential of pep3 and pep3mut was determined at a N/P ratio 20 and revealed positively charged particles (42 ± 6 mV) with a suitable size for gene delivery (163 ± 20 nm). These observations correspond to the findings reported by Fabre et al, stating that pDNA condensation should be feasible from 6 or more positive charges per peptide, leading to particles in non-ionic isotonic solutions of around 80 nm [18]. It should be noted, however, that particle size will dramatically depend on the ionic composition of the solution, leading to pDNA/peptide particles of more than 600 nm in for example optiMEM® used to perform the transfections.

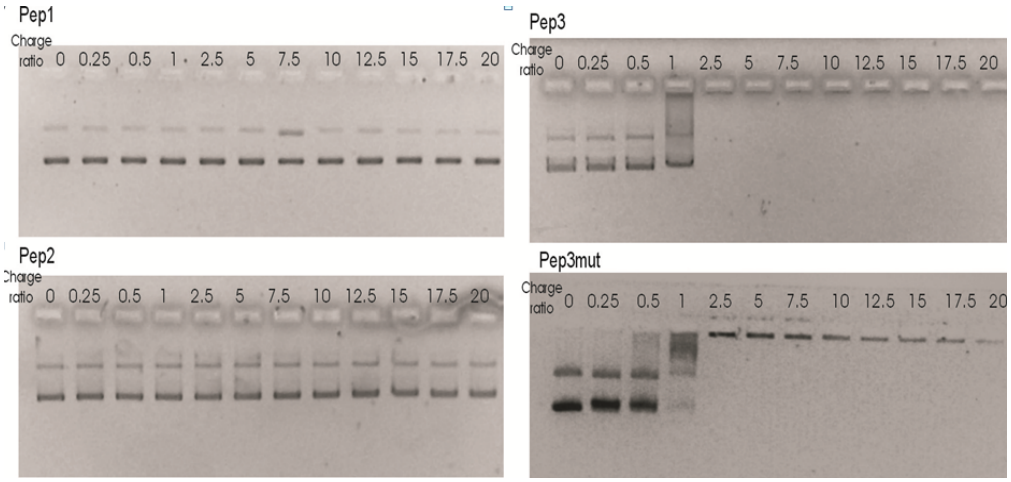


Fig. 3. Gel retardation assay of pep1, pep2, pep3 and pep3mut in function of N/P ratio of the pDNA/peptide complexes in low salt buffer (20 mM Hepes). Each well contained 0.2 µg of pDNA. N/P ratio 0 corresponds to non-complexed pDNA.

To test the ability of cells to specifically phosphorylate pep3/pDNA complexes at the onset of cell division, cell extract was prepared from non synchronized HeLa cells or from synchronized HeLa cells in the G2/M phase. pep3/pDNA complexes with N/P ratio 5, 10 and 20 were incubated in buffer or in both types of cell extract. As control, also free pDNA or pDNA incubated with cell extract from synchronized cells was loaded. Figure 4 demonstrates that only pep3/pDNA complexes incubated with cell extract from synchronized cells show release of plasmid DNA, especially for the lower N/P ratios 5 and 10. This indicates that the peptides are indeed phosphorylated by Cdk1 and partially loose their pDNA binding capacity. It should be noted that release is not complete, as pep3/pDNA complexes can still be seen in the wells of the gel. Also, cell lysate mildly degrades the pDNA that is released as can be seen from the increased presence of the open circular form (compared to pDNA in lane 1). In the presence of buffer or cell extract from non-synchronized cells, pDNA release was not observed.

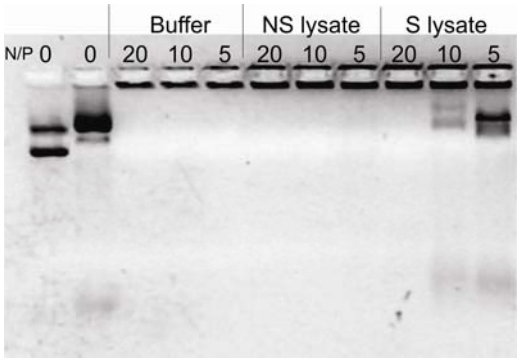


Fig. 4. Gel electrophoresis of free pDNA in buffer (Lane 1) or incubated with cell extract from synchronized Hela cells (Lane 2). pep3/pDNA complexes with the given N/P ratio were incubated in buffer (lane 3-5), cell extract from non-synchronized HeLa cells (lane 6-8, NS lysate) or cell extract from synchronized HeLa cells (lane 9-11, S lysate) at 37°C during 2 hours. Only cell lysate from synchronized cells partially displays the pDNA from the pep3/pDNA complexes, which is most likely attributed to the elevated Cdk1 activity.

Cellular delivery of pDNA/peptide complexes

Unfortunately, pDNA/peptide complexes alone were not capable of generating GFP positive cells. Suprisingly, pDNA/peptide complexes were not taken up by cells, despite their small size and positive charge (data not shown). Also, our pDNA/peptide complexes most likely lack endosomal escape properties, as they do not contain histidines in their sequence. Histidine is not protonated at neutral pH, but is at lower pH. The presence of histidine provides synthetic peptides with buffering capacity, leading to the so called proton sponge effect and endosomal escape of the pDNA/peptide nanoparticles [18].

A possible strategy to induce uptake and endosomal escape of our pDNA/peptide complexes is the delivery with lipid based carriers, that are known to efficiently escape the endosomal compartment by lipid mixing [20]. Therefore, we evaluated the formation of ternary complexes in which the pDNA was precondensed by the cationic peptides and then incubated in opiMEM® containing a certain amount of the commercially available lipid carrier RNAimax®. In this way, (pDNA/peptide)/lipid complexes are formed. Figure 5 shows a typical confocal image of HeLa cells treated with these ternary complexes. Uptake in the cells is clearly visible by the red dotted structures inside the cytoplasm of the cells, representing pDNA/peptide complexes in endosomal vesicles.

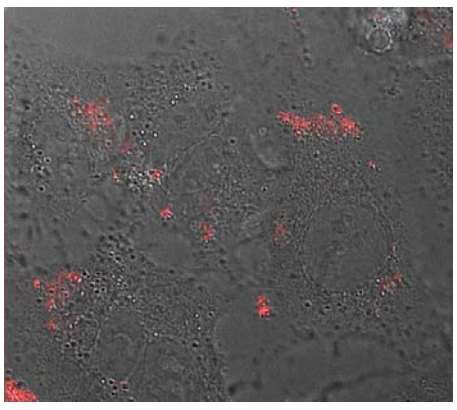


Fig. 5. Confocal images of HeLa cells transfected with Cy5-pDNA/pep3 complexes at N/P ratio 20.

Fig 6 shows the transfection efficiencies obtained with different amounts of RNAimax® added to pDNA alone or pDNA precomplexed with pep3 at N/P ratio 10. The transfection efficiencies were always compared to pDNA/lipid complexes where the pDNA was not precondensed with peptides

before adding the lipids (e.g. the usual transfection procedure). First of all, it can be seen that the transfection efficiency increases in function of the added amount of RNAimax®. Second, the beneficial effect of precondensing pDNA with pep3 is only significantly present when low amount of RNAimax® was used, such as 0.5 or 1 µl per µg of pDNA. From 2 or 3 µl RNAimax® per µg pDNA, pep3 has no beneficial effect on transfection efficiencies. As also toxicity increased with this higher amount of RNAimax®, we choose to perform further experiments with the combination (pDNA/pep3)/RNAimax® at the amount of 1 µl of RNAimax® per µg of delivered plasmid.

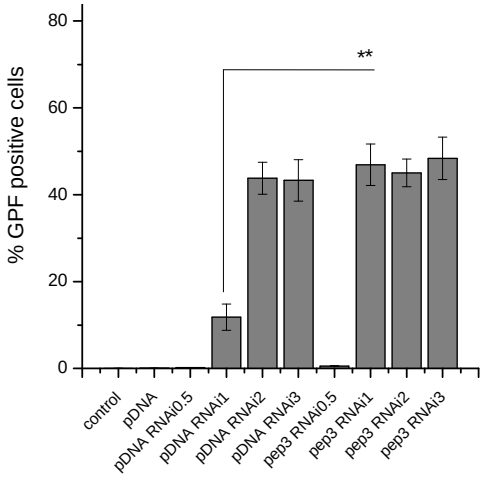


Fig 6. Transfection efficiency in HeLa cells of pDNA/lipid complexes and (pDNA/peptide)/lipid ternary complexes. As commercially available lipid- based carrier, RNAimax® (RNAi) was used in the ratio 0.5, 1, 2 or 3 µl of carrier for 1 µg of pDNA. pDNA stands for pDNA/lipid complexes, while pep3 stands for pDNA precondensed with pep3 before adding the RNAimax®, leading to ternary (pDNA/pep3)/lipid complexes. Significant differences were determined by a student t-test. P values were < 0.001 (**).

Effect of cell division on phosphorylation responsive peptides in HeLa and A549 cells

In a next set of experiments, transfections were performed with pDNA precondensed with peptides that can (pep1, pep2 and pep3) or cannot (pep3mut) be phosphorylated by Cdk1 at the onset of cell division. pDNA alone or pDNA precondensed with peptides were delivered to the cells by the lipid carrier RNAimax®. The percentage of GFP positive cells and the average fluorescence intensity per cell are depicted in Figure 7. In HeLa cells, the percentage of GFP positive cells (Fig 7A) is not significantly different between pDNA delivered by RNAimax®, or pDNA precondensed with the peptides pep1 and pep2 and subsequently delivered by RNAimax®. This can most likely be attributed to the poor DNA condensing properties of pep1 and pep2 as seen in Fig 3. When the pDNA is precondensed with pep3 or pep3mut, however, transfection increases from 50 to 70% in non-

synchronized HeLa cells (Fig 7A, black bars) or from 60 to 90% in synchronized HeLa cells (Fig 7A, grey bars). A difference between pep3 and pep3mut can however not be found, demonstrating that it is not the phosphorylation responsive feature of pep3 that is predominantly responsible for this effect. Interestingly, not only the percentage of GFP positive cells, but also the mean fluorescence intensity per cell increases 3-fold in non-synchronized HeLa cells and even 7-fold in synchronized HeLa cells (Fig 7B). This demonstrates that precondensation of pDNA helps more pDNA to reach the nuclear interior. This could result from a more efficient trafficking of the compacted pDNA/peptide complexes to the perinuclear region and/or the protection of pDNA in the cytoplasmic environment. For N/P ratio 5 in synchronized HeLa cells, the average fluorescent intensity is significantly higher for pep3 when compared to pep3mut (p value < 0.05). This corresponds to the observation that phosphorylation of pep3 at N/P ratio 5 did indeed help more pDNA to release from the carrier. Although statistically different, the effect is however small and could not be observed in non-synchronized HeLa cells, leading us to conclude that the effect of phosphorylation of pep3 is not as beneficial as what we had hoped for. It should be noted that also upon electroporation of plasmid DNA complexes prepared with pep3 or pep3mut, no differences in the percentage of transfected cells was observed (data not shown).

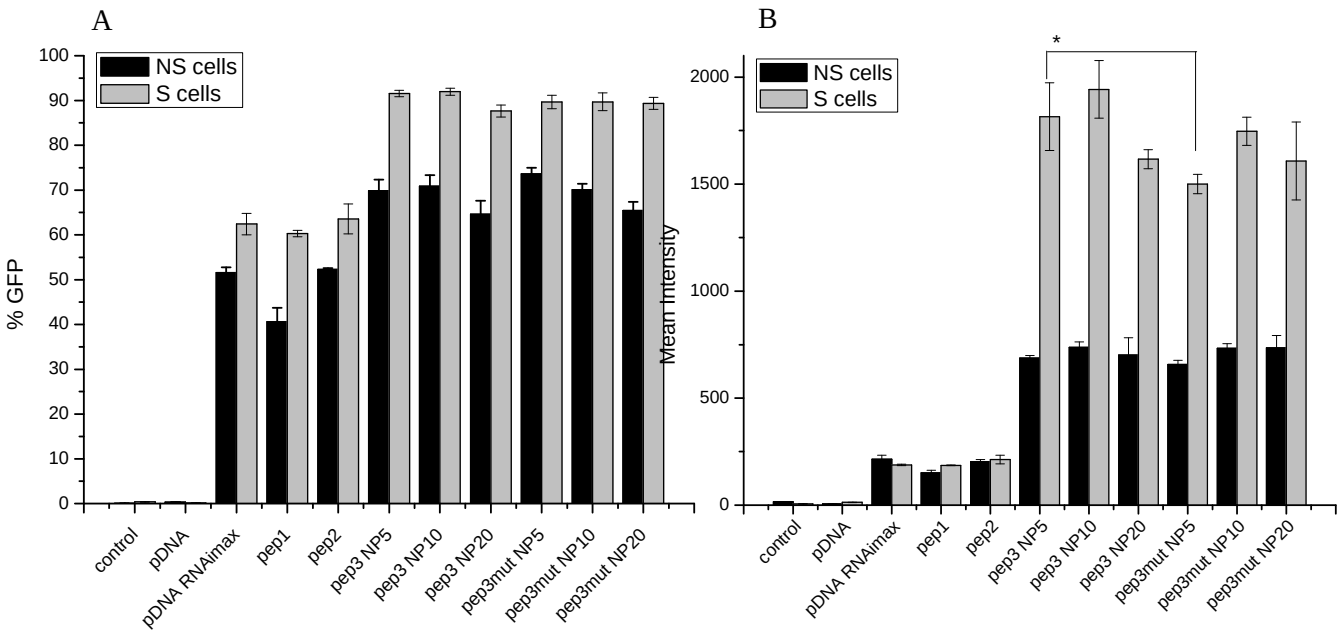


Fig. 7. Percentage of GFP positive **HeLa** cells (A) and mean fluorescence intensity per cell (B) for pDNA delivered by RNAimax® in free form (pDNA RNAimax) or precondensed with pep1 and pep2 (N/P ratio 20) and pep3 and pep3mut with N/P ratio 5, 10 or 20. Transfections were performed in non-synchronized cells (NS cells, black bars) and synchronously dividing cells (S cells, grey bars). Significant differences were determined by a student t-test. * denotes a P value < 0.05.

Figure 8 shows the same set of experiments performed in A549 cells. It can be noted that the absolute numbers of percentage of GFP positive cells and mean fluorescence intensity per cell are much lower when compared to HeLa cells. Again, pep1 and pep2 did not give additional benefits to pDNA delivered by RNAimax®. Using pep3 and pep3mut, several observations can be made. First of all, both the percentage of GFP positive cells and the average fluorescence intensity decreased with increasing N/P ratios. In non-synchronized A549 cells, the effect of pep3 was however significantly higher when compared to pep3mut at N/P ratio 5 (Fig 8A, black bars). This again corresponds to figure 4, where phosphorylation at N/P ratio 5 did have an effect on enhancing the release of pDNA from the carrier. In synchronized cells, however, no major differences were observed between pep3 and pep3mut (Fig 8A, grey bars). Although both of them gave higher transfection efficiencies when compared to pDNA delivered by RNAimax® alone, phosphorylation of pep3 does not seem to play a major role in enhancing the transfection efficiency. It is however worth taking a look at the average fluorescence intensities per cell, giving an idea of the amount of pDNA that reaches the nuclear interior (Fig 8B). Both in non-synchronized and especially in synchronized A549 cells the average fluorescence intensity is higher when compared the pDNA/RNAimax®. As in the HeLa cells, this points out that precondensation of pDNA adds some beneficial features such as improved intracellular trafficking and/or stability of the nucleic acids. In non-synchronized cells, pep3 at N/P 5 is beneficial over pep3mut. In synchronized cells, pep3 at N/P10 and N/P 20 are beneficial over pep3mut. Although significant (p value < 0.05), the use of pep3 over pep3mut only shows slight improvement in transfection efficiency, leaving us to conclude that phosphorylation of pep3 helps, but is not the main determinant for improved pDNA delivery when compared to pDNA/RNAimax® alone.

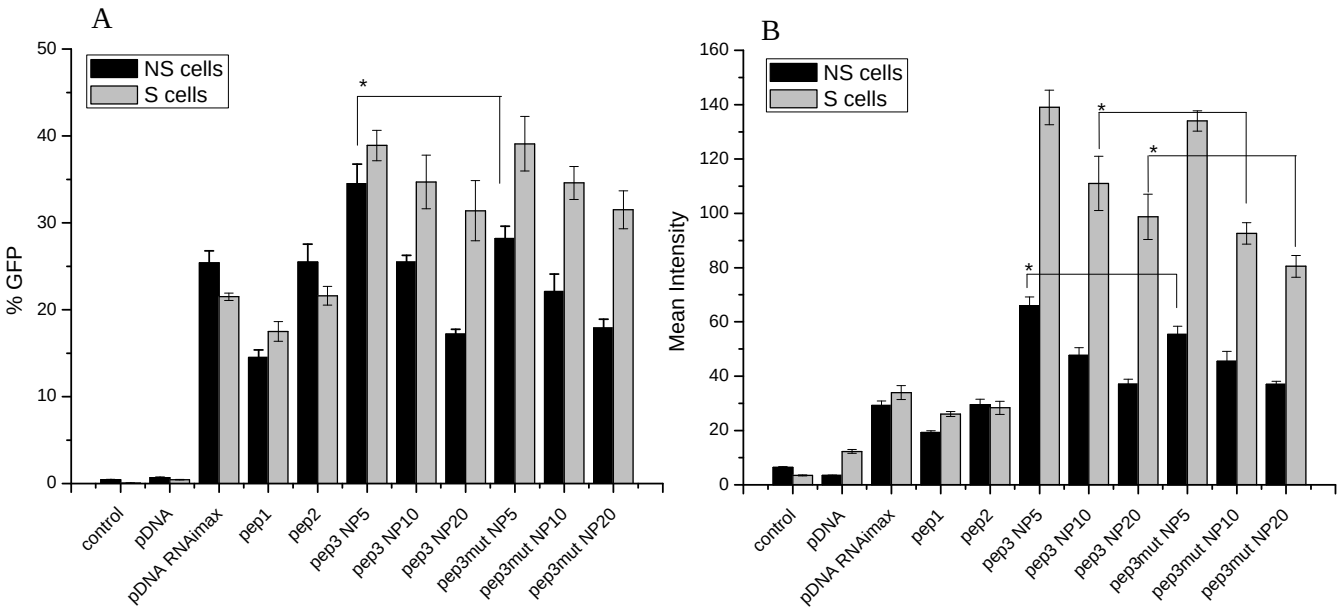
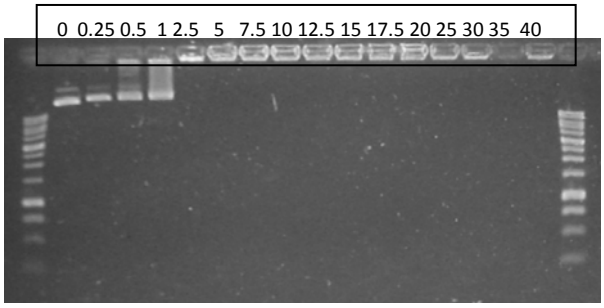


Fig. 8. Percentage of GFP positive **A549** cells (A) and mean fluorescence intensity per cell (B) for pDNA delivered by RNAimax® in free form (pDNA RNAimax) or precondensed with pep1 and pep2 (N/P ratio 20) and pep3 and pep3mut with N/P ratio 5, 10 or 20. Transfections were performed in non-synchronized cells (black bars) and synchronously dividing cells (grey bars). Significant differences were determined by a student t-test. * denotes a P value < 0.05.

Characterization of chromatin targeting peptides

In the second strategy, we evaluated the ability of synthetic peptides containing AT-hook sequences (containing 21 positive charges per mol peptide, further called 'AT') or histone binding properties (containing 14 positive charges per mol peptide, further called 'His') to target pDNA/peptide complexes to the chromatin during cell division. As control, ODL (10 D-lysines) and OLL (20 lysines) were used. Gel retardation assays demonstrated that both AT and His complexed pDNA starting from N/P ratio 2.5 (supplemental fig 1).



Suppl Fig 1. Gel retardation assay of His in function of N/P ratio of the pDNA/peptide complexes. Each well contained 0.2 µg of pDNA. N/P ratio 0 corresponds to non-complexed pDNA.

Figure 9 shows the size and zetapotential of different pDNA/peptide complexes in 20 mM Hepes buffer. Both ODL and OLL show large aggregates at N/P ratio 10. At N/P ratio 20, ODL still shows large complexes while OLL has decreased to about 200 nm complexes. AT and His form complexes of about 100 nm both at N/P ratio 10 and 20. All complexes have a positive zetapotential between 4 and 12 mV.

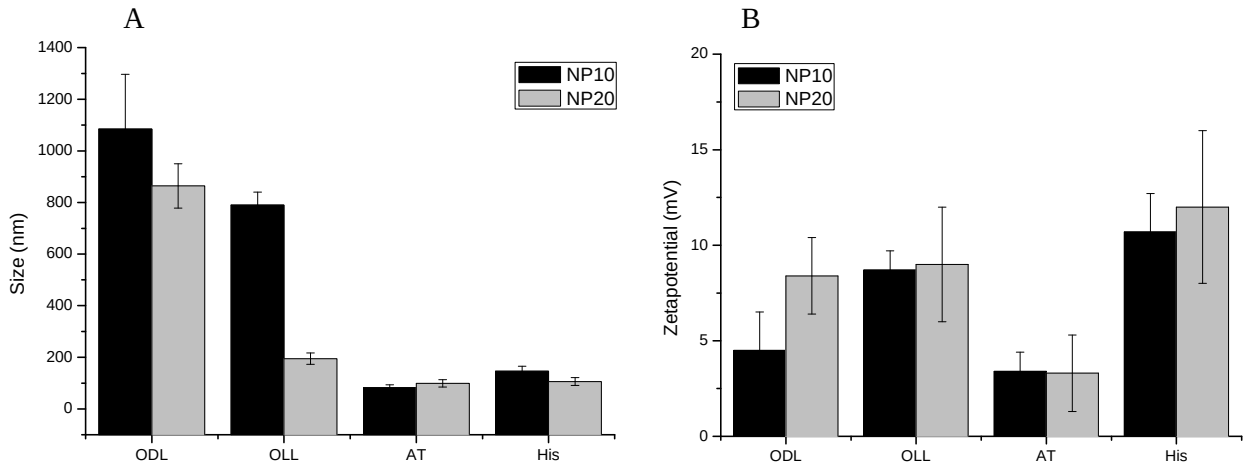


Fig. 9. Size (A) and zetapotential (B) of pDNA/peptide complexes at N/P ratio 10 and 20 in 20 mM Hepes buffer.

To assess the chromatin targeting, the Xenopus nuclear envelop reassembly (XNER) assay was performed. Therefore, complexes were incubated with chromatin *in vitro* and the number of particles bound to chromatin was calculated (Fig 10). It can be seen that the chromatin binding increases in the order His < ODL < AT < OLL. This pattern seems to follow the charge density of the different synthetic peptides (e.g. 12, 10, 21 and 20), rather then the chromatin targeting properties of His and AT.

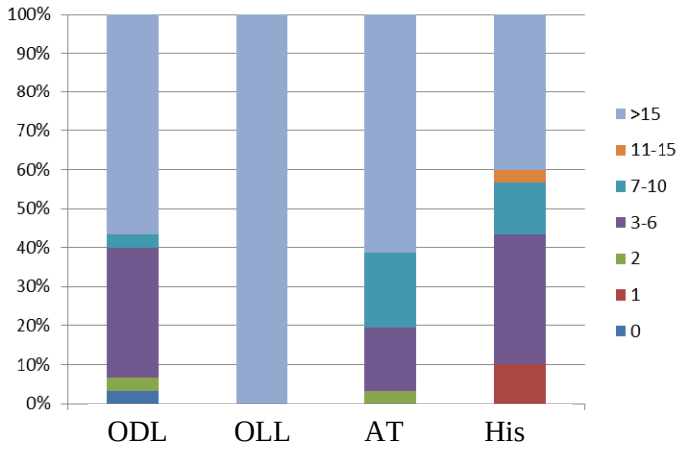


Fig. 10. Chromatin binding of pDNA/peptide particles made with pepetides ODL, OLL, AT and His. The number of particles per chromatin strand was determined with the Xenopus nuclear envelope reassembly (XNER) reaction.

Direct cytoplasmic delivery of chromatin targeting pDNA/peptide complexes

In a first experiment, we aimed to directly bring the pDNA/peptide complexes into the cytoplasm of the cells by electroporation. This circumvents the need for endosomal escape so that the chromatin targeting effect of the peptides can not be masked by differences in uptake and/or endosomal escape efficiency. Figure 11 shows the uptake (grey bars) and percentage of GFP positive cells (black bars) after electroporating free pDNA or pDNA/peptide complexes into non-synchronized HeLa cells. For most pDNA/peptides complexes, about 60% of the cells have taken up the complexes. Only the pDNA/AT complexes were electroporated more efficiently, as 90% of the cells contained the complexes. When looking at the transfection efficiencies, however, electroporation of free pDNA always generated the best results, followed by AT and His. This already demonstrates that the chromatin targeting effect does not seem to help pDNA to reach the nuclear interior in a asynchronously dividing cell culture. It should be noted that upon electroporation of pDNA/peptide complexes made with pep3 and pep3mut, the same uptake and transfection efficiency was seen as with pDNA/peptide complexes made with AT (data not shown). As both pep3 and pep3mut do not contain specific chromatin targeting sequences, this indicates that not only chromatin targeting is responsible for the observed effect.

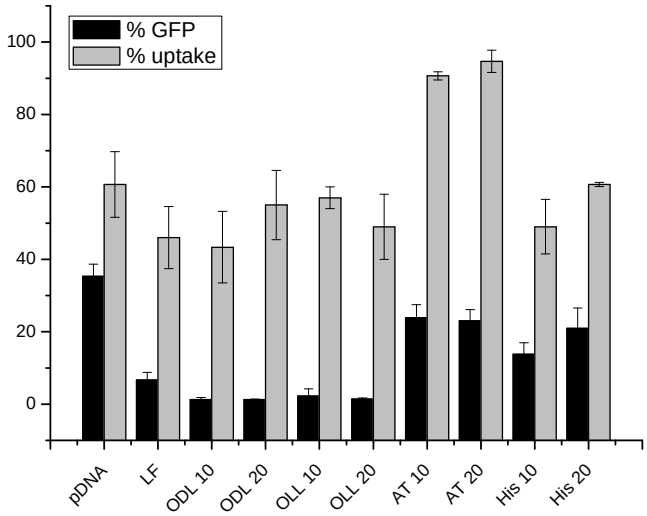


Fig. 11. Percentage of GFP positive HeLa cells (black bars) and uptake (grey bars) of pDNA/peptide complexes following electroporation. pDNA was electroporated free, or after precondensation with synthetic peptids at N/P ratio 10 or 20. As control, also pDNA complexed with lipofectamine2000® was used.

Lipofection of chromatin targeting pDNA/peptide complexes

In the next set of experiments, again ternary (pDNA/peptide)/RNAimax® complexes were used to deliver the pDNA/peptide complexes into the cytoplasm of the cells. The percentage of GFP positive cells and the mean fluorescence intensities per cell were determined for asynchronously dividing cells, synchronously dividing cells and arrested (non-dividing) cells (Fig. 12). When compared to pDNA/RNAimax®, the ternary (pDNA/peptide)/RNAimax® complexes that performed significantly better (p values < 0.05) are denoted with an asterix. It can be noted that especially in synchronized cells, AT and His perform better in transfecting a higher amount of the cell population (Fig. 12A, grey bars). Also, a higher amount of GFP copies per cell was found (Fig 12B, grey bars). In non-synchronized cells the amount of GFP positive cells is more or less equal (Fig 12A, black bars), but the mean fluorescence intensity per cell is again significantly higher when compared to pDNA/RNAimax® (Fig 12B, black bars). Both AT and His thus add beneficial effects to the pDNA when delivered in non-synchronized or synchronized cells. The same beneficial effect can however be noted for OLL at N/P ratio 20, demonstrating that the chromatin targeting effect of AT and His is not the main reason for the improved transfection efficiency. More likely, the physicochemical properties like size and charge of the pDNA/peptide complexes determine their intracellular performance, rather than the specific

cell responsive properties that were assigned to the synthetic peptides. Interestingly, both OLL20 and AT20 showed good transfection properties in arrested HeLa cells, indicating that they aid the delivery of pDNA to the nuclear compartment through the NPCs to some extent. It has been reported before that oligolysines with 16 lysine residues ((Lys)16) could indeed improve nuclear translocation [18]. This effect is apparently also true for the AT-hook derived sequence used in this study.

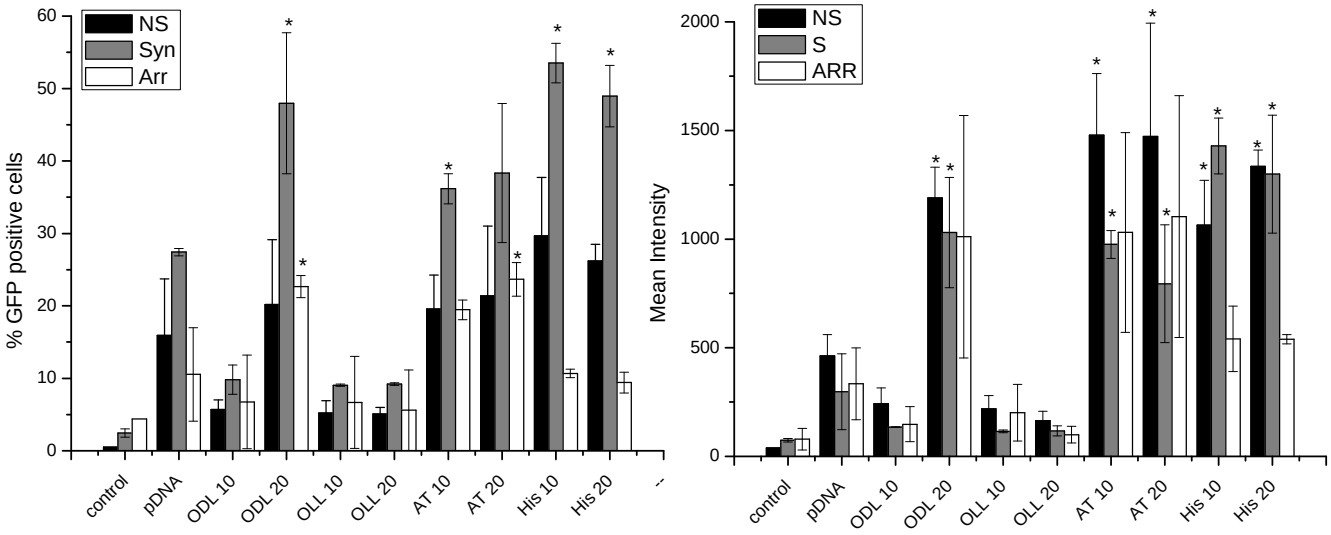


Fig. 12. Percentage of GFP positive HeLa cells (A) and mean fluorescence intensity per cell (B) for pDNA delivered by RNAimax® in free form (pDNA) or precondensed with synthetic peptides ODL, OLL, AT and his at N/P ratio 10 or 20. Transfections were performed in non-synchronized cells (black bars), synchronously dividing cells (grey bars) and arrested (non-dividing) cells (white bars). Significant differences were determined by a student t-test. * denotes a P value < 0.05.

CONCLUSION

The efficient delivery of plasmid DNA to the nucleus of cells remains a challenge. Currently, cells are challenged with an excessive amount of pDNA to reach *in vitro* gene delivery. We and others, however, found that more than 90% of the originally administered pDNA can be replaced by non-coding pDNA, demonstrating the enormous potential to further optimize pDNA delivery and decrease the dose applied to cells [21, 22]. In this research paper, we evaluated two strategies to increase the fraction of initially applied pDNA that reaches the nuclear interior of the cells. The first strategy explored the possibility of releasing pDNA specifically at the onset of cell division by using phosphorylation responsive peptides, recognized by Cdk1. It was already suggested by Wilke et al.

that peptides containing the recognition sequence of Cdk1 depend on mitosis for efficient gene delivery [23]. They hypothesized that phosphorylation of the peptide at the onset of cell division indeed diminishes the pDNA binding properties, thereby leading to more efficient transfection. A peptide control, lacking the phosphorylation sequence, was however not used. In our study, the phosphorylation responsive peptide Pep3 also performed significantly better in dividing cells, especially when the average amount of expressed GFP per cell is considered (Fig. 6B and 8B). This result seemed very promising. However, when Pep3mut (lacking the phosphorylation sequence) was used to condense the pDNA, almost the same effect was observed. Therefore, phosphorylation of Pep3 by Cdk1 does not seem to be the main reason for the improved transfection efficiency, although it does help to some extent at the lower N/P ratios, for which pDNA release was indeed also observed in cell extract from synchronized cells.

In a second strategy, we aimed to target pDNA/Peptide complexes to the chromatin during cell division. We demonstrated previously that chromatin targeting leads to a 2-3 fold increase in nuclear inclusion using the *in vitro* XNER assay [14]. In this study, peptides containing an AT-hook (AT) or the chromatin targeting sequence from the herpes virus LANA were used (His). As controls, non-targeted oligo-L-Lysine (OLL) or oligo-D-lysine (ODL) was used. The XNER assay already pointed out that the chromatin targeting properties of AT and His were in the same range as for OLL and ODL respectively (Fig 9). Also in living cells, the chromatin targeting sequence were not significantly better than their non-targeted controls. Upon electroporation, precondensation of pDNA with any kind of peptide even diminished the transfection efficiency. It should be noted that it is possible that the electroporation procedure also disturbes the nuclear membare, directly bringing the pDNA into the nucleus of the cells even without cell division. In this case, it is logical that precondensation of pDNA is not beneficial as the pDNA then first needs to dissociate from its peptide carrier before nuclear transcription can start. Interestingly, also precomplexation of pDNA with lipofectamine2000® is not beneficial. It is indeed know that lipid based carriers need endocytosis and endosomal escape to efficiently release the complexed DNA into the cytoplasm of the cells [24-26]. When ternary

(pDNA/peptide)/RNAimax® complexes were used to deliver the pDNA/peptide complexes into the cytoplasm of the cells, again precondensation of pDNA with peptides improved the transfection efficiency compared to pDNA delivery by RNAimax®. Also the OLL control, however, improved transfection efficiency and a clear correlation with the chromatin targeting motifs of the peptides AT and His could not be found.

In conclusion, at a ratio of 1 µl RNAimax per µg of plasmid DNA, transfection efficiency of plasmid DNA can be improved when ternary (plasmid/peptide)/RNAimax® complexes are applied rather than (suboptimum) pDNA/RNAimax® complexes alone. It does seem, however, that this effect rather stems from a better condensation of pDNA by the peptides, leading to an improved intracellular mobility or an enhanced protection against enzymatic degradation, rather than the phosphorylation properties of Pep3, or the chromatin targeting properties of AT and His. Nevertheless, we do believe there is a future for gene carriers that are responsive to intracellular signaling, as has been demonstrated for e.g. caspase-3 signaling [27] and cyclic AMP-dependent protein kinase (PKA) [28-30]. In these systems, the kinase or caspase recognition sequences (respectively ALRRASLG for PKA ("PAK" system) and AGDEVDGKKKKKK for caspase-3 ("PAC" system)) are grafted as side chains on an acrylamide backbone. For the PAC system, the caspase-3 enzyme cleaves the recognition sequence, resulting in release of the KKKKKK part of the peptide. Therefore, plasmid DNA binding properties are lost upon cleavage. For the PAK system, protein kinase A phosphorylates each side chain, resulting in the release of plasmid DNA. The principle behind plasmid DNA release is thus comparable to our approach by using pep3. Some major differences, however, can be noted. First, the peptide side chains only contain 2 positive charges, compared to 15 positively charged amino acids in our pep3. Upon phosphorylation, respectively 1 and 3 negative charges are introduced in the PAK system or the pep3. Therefore, the charge density changes from 2 to 1 or 15 to 12 respectively. As could be seen in Figure 4, phosphorylation of pep3 results in partial pDNA release, especially at lower N/P ratios. With our present knowledge, we believe complete pDNA release can only be obtained when the used peptides contain less than 6 positive charges after phosphorylation. Decreasing the number of lysines

in our pep3 sequence, could thus further increase the effect of phosphorylation during cell division. Another important difference, is that the non-phosphorylated version of the PAK system completely prevents plasmid DNA transcription. Phosphorylation can 'undo' the inhibitory effect of the PAK polymer, but does not increase transcription when compared to a control vector for pDNA delivery. As our non-phosphorylated control peptide (pep3mut) does not prevent the transcription of pDNA, the differences observed upon phosphorylation when compared to an already efficient control are not that clear. Finally, in living cells the phosphorylation of the PAK system was only effective in cells with an upregulated expression profile of PKA, such as for example certain cancer types, or chemically stimulated by the use of forskolin. Our system is intended to respond to the normal levels of Cdk1 activity during mitosis. Figure 4 demonstrated that these levels do result in partial plasmid DNA release from the pep3, especially at lower N/P ratios. For future strategies, it could be interesting to evaluate the peptide RGDSPRRSPRRSPRR, which contains the minimal of 6 positive charges for pDNA condensation, but contains 3 phosphorylation sides, thereby decreasing the charge density by 50% upon Cdk1 activity. It should be noted that as in our study, the PAK system did also need an extra vector to be able to transfect living cells, as the pDNA/peptide particles alone were not taken up in living cells. Therefore, the use of ternary complexes to assure cellular uptake of cell responsive peptides should always be considered.

Regarding strategy 2, it should be noted that the chromatin targeting properties are lost as soon as the pDNA releases from the peptide carrier. Therefore, it would be more interesting to couple the chromatin targeting peptides to the pDNA itself, rather than incorporating them into the carrier sequence. Eventually, one might even consider combining strategy 1 and strategy 2 to further improve pDNA delivery, resulting in partial or complete DNA release by kinase activity for improved nuclear entry but keeping the chromatin attaching ability by using for example pDNA equipped with chromatin targeting sequences.

ACKNOWLEDGMENTS

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FIGURES

Figure 1

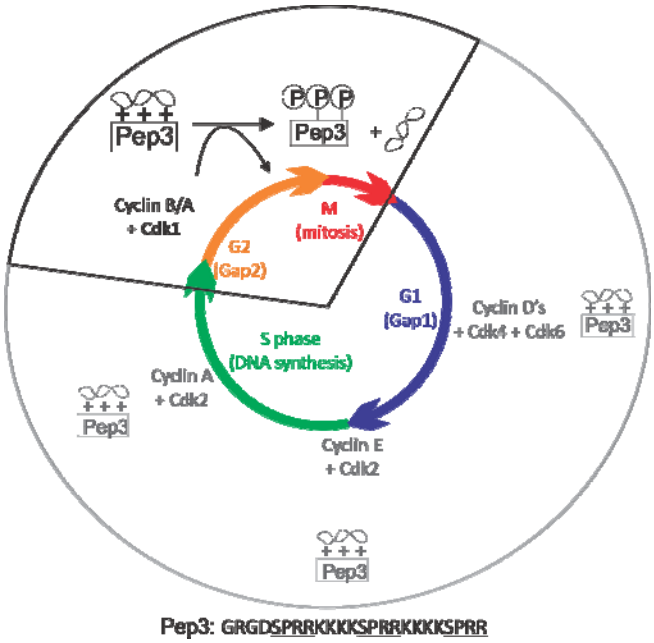


Fig. 1. Strategy 1: phosphorylation responsible peptides (pep3) for specific release of pDNA at the onset of cell division (M: mitosis). During Gap 1 (G1), DNA synthesis (S phase) and Gap 2 (G2 phase), pep3 is not phosphorylated and is expected to stay in complex with plasmid DNA. At the onset of cell division, Pep3 is phosphorylated by Cdk1 at the three recognition sites, leading to less affinity to pDNA and release of pDNA at the beginning of M phase.

Figure 2

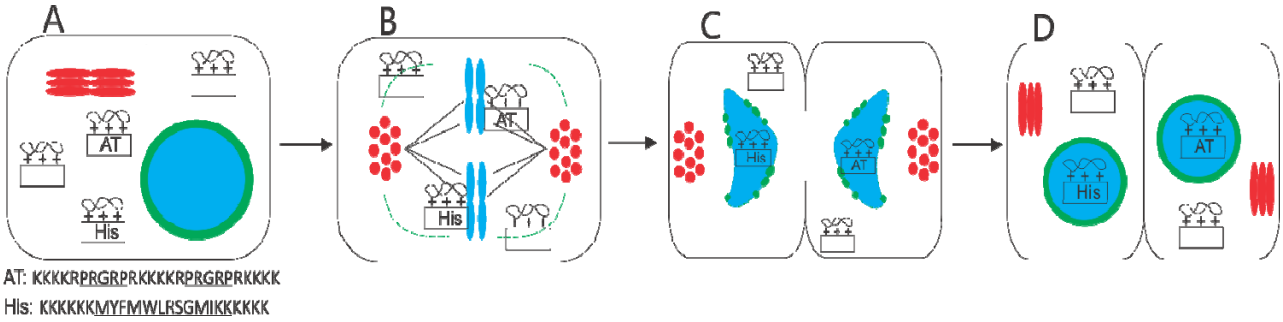


Fig 2. Strategy 2: peptides with chromatin targeting properties (AT and His) are used to bind plasmid DNA (A). During cell division, the nuclear envelop (green) is temporarily disassembled and the complexes can anchor to the chromatin (blue) (B). When the daughter nuclei are formed, targeted complexes are entrapped in the nuclear interior, while non-targeted complexes are not (C-D). Red = golgi apparatus.

Figure 3

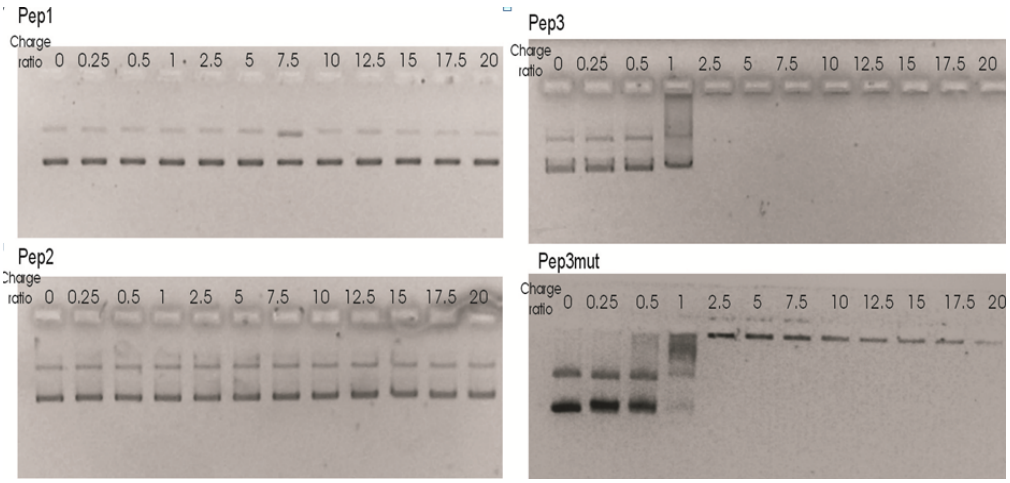


Fig. 3. Gel retardation assay of pep1, pep2, pep3 and pep3mut in function of N/P ratio of the pDNA/peptide complexes in low salt buffer (20 mM Hepes). Each well contained 0.2 µg of pDNA. N/P ratio 0 corresponds to non-complexed pDNA.

Figure 4

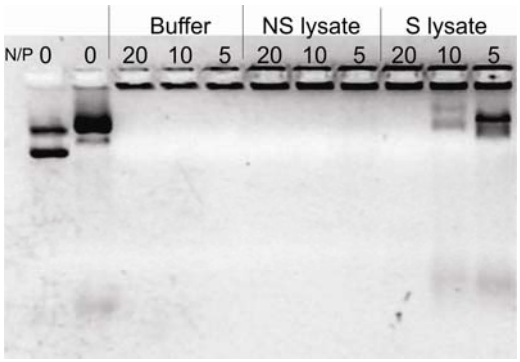


Fig. 4. Gel electrophoresis of free pDNA in buffer (Lane 1) or incubated with cell extract from synchronized Hela cells (Lane 2). pep3/pDNA complexes with the given N/P ratio were incubated in buffer (lane 3-5), cell extract from non-synchronized HeLa cells (lane 6-8, NS lysate) or cell extract from synchronized HeLa cells (lane 9-11, S lysate) at 37°C during 2 hours. Only cell lysate from synchronized cells partially displays the pDNA from the pep3/pDNA complexes, which is most likely attributed to the elevated Cdk1 activity.

Figure 5

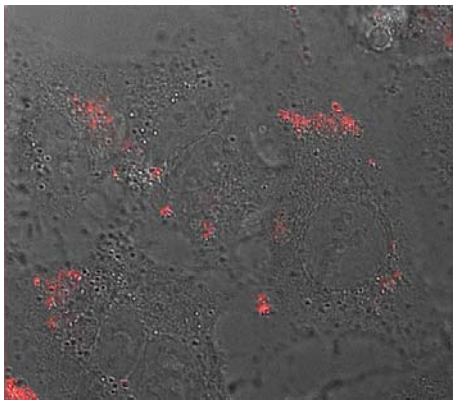


Fig. 5. Confocal images of HeLa cells transfected with Cy5-pDNA/pep3 complexes at N/P ratio 20.

Figure 6

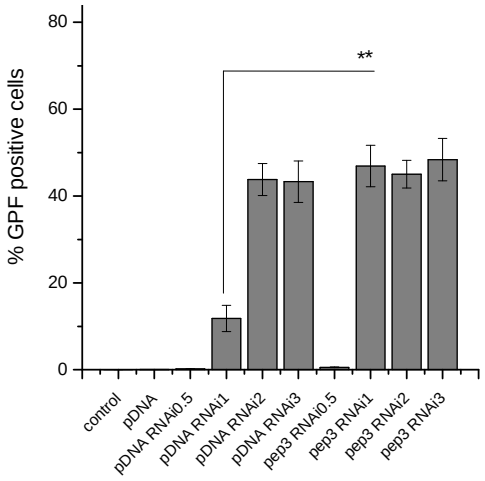


Fig 6. Transfection efficiency in HeLa cells of pDNA/lipid complexes and (pDNA/peptide)/lipid ternary complexes. As commercially available lipid- based carrier, RNAimax® (RNAi) was used in the ratio 0.5, 1, 2 or 3 µl of carrier for 1 µg of pDNA. pDNA stands for pDNA/lipid complexes, while pep3 stands for pDNA precondensed with pep3 before adding the RNAimax®, leading to ternary (pDNA/pep3)/lipid complexes. Significant differences were determined by a student t-test. P values were < 0.001 (**).

Figure 7

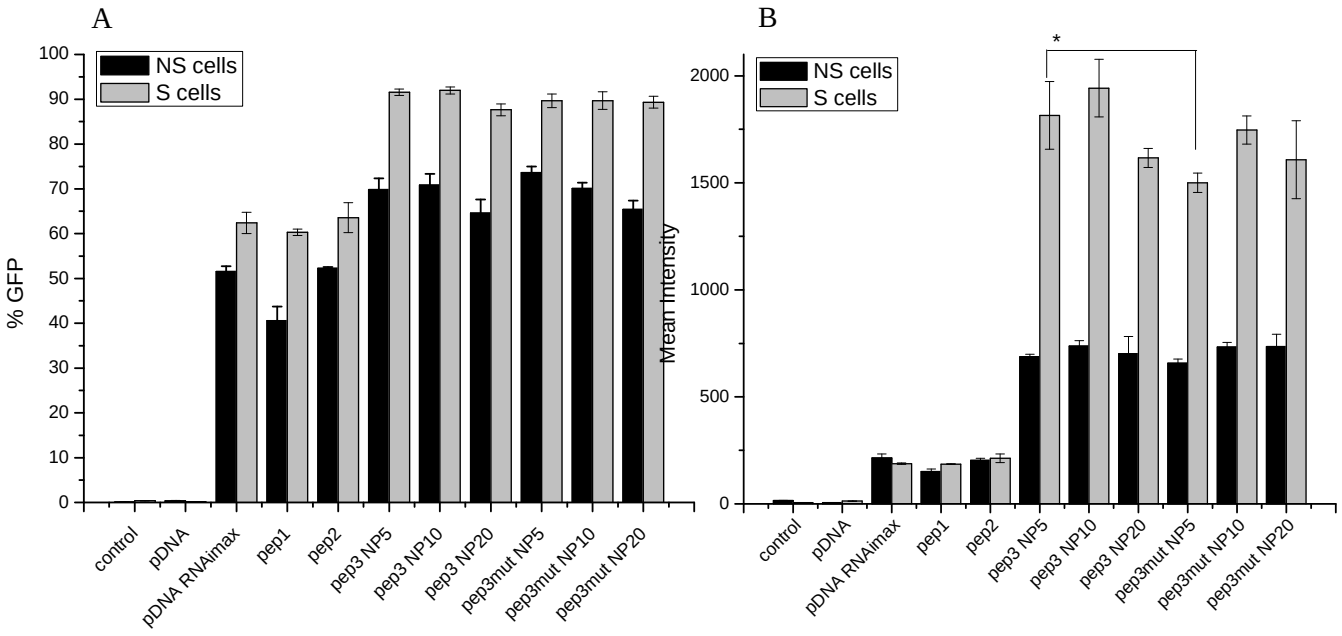


Fig. 7. Percentage of GFP positive **HeLa** cells (A) and mean fluorescence intensity per cell (B) for pDNA delivered by RNAimax® in free form (pDNA RNAimax) or precondensed with pep1 and pep2 (N/P ratio 20) and pep3 and pep3mut with N/P ratio 5, 10 or 20. Transfections were performed in non-synchronized cells (black bars) and synchronously dividing cells (grey bars). Significant differences were determined by a student t-test. * denotes a P value < 0.05.

Figure 8

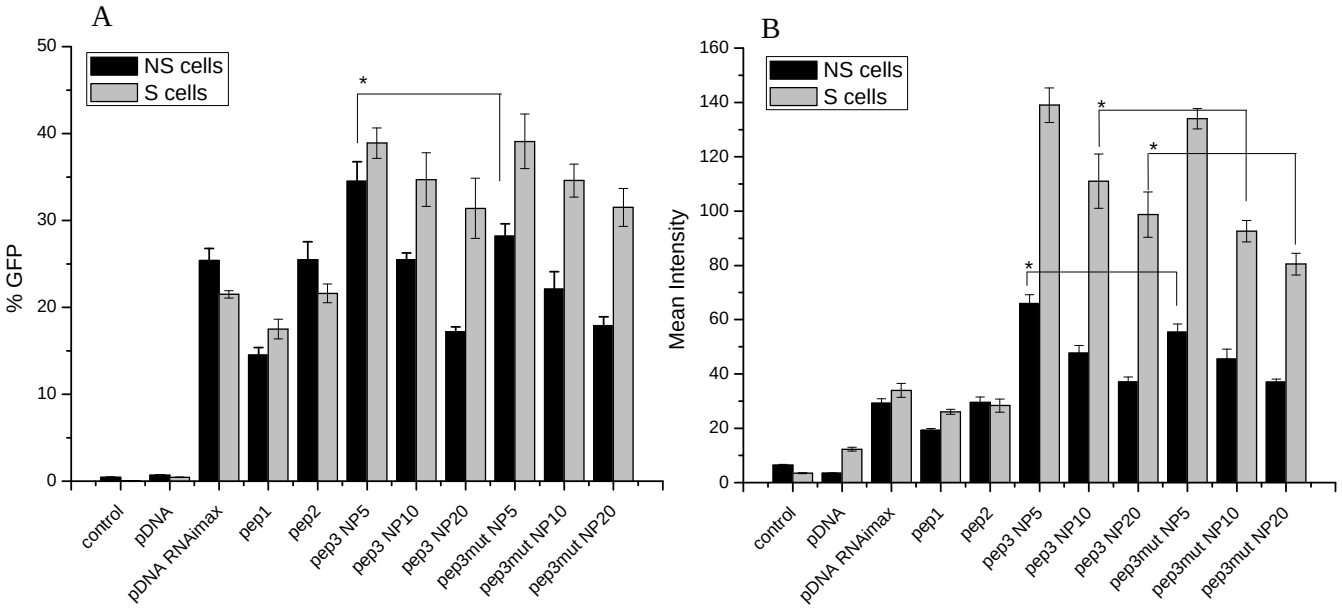


Fig. 8. Percentage of GFP positive **A549** cells (A) and mean fluorescence intensity per cell (B) for pDNA delivered by RNAimax® in free form (pDNA RNAimax) or precondensed with pep1 and pep2 (N/P ratio 20) and pep3 and pep3mut with N/P ratio 5, 10 or 20. Transfections were performed in non-synchronized cells (black bars) and synchronously dividing cells (grey bars). Significant differences were determined by a student t-test. * denotes a P value < 0.05.

Figure 9

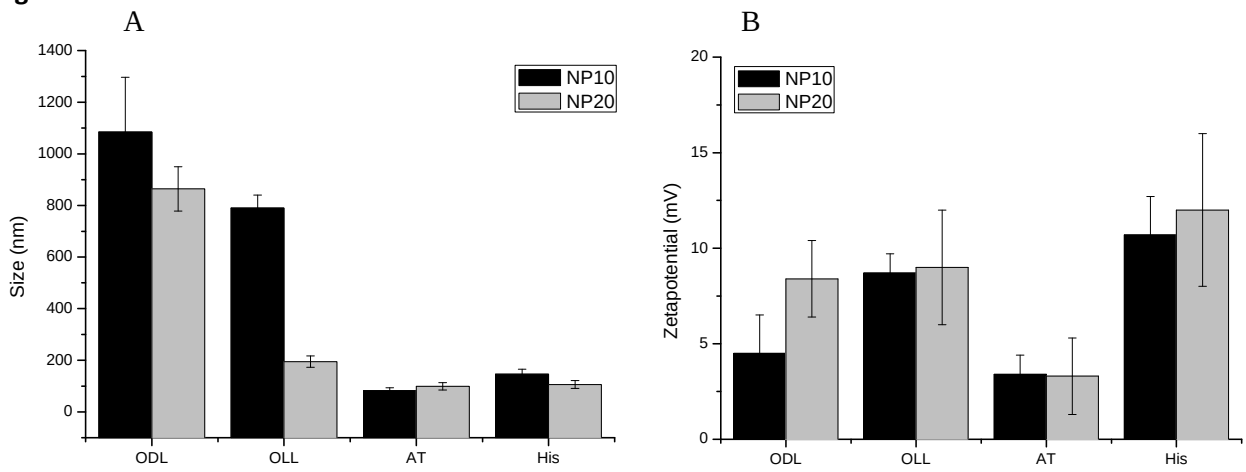


Fig. 9. Size (A) and zetapotential (B) of pDNA/peptide complexes at N/P ratio 10 and 20 in 20 mM Hepes buffer.

Figure 10

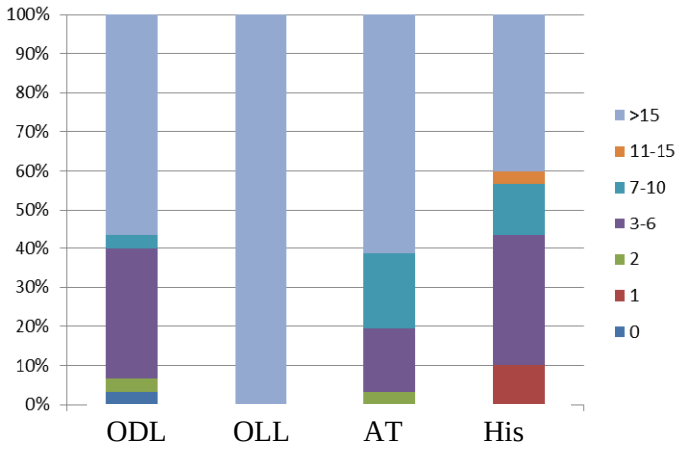


Fig. 10. Chromatin binding of pDNA/peptide particles made with peptides ODL, OLL, AT and His. The number of particles per chromatin strand was determined with the Xenopus nuclear envelope reassembly (XNER) reaction.

Figure 11

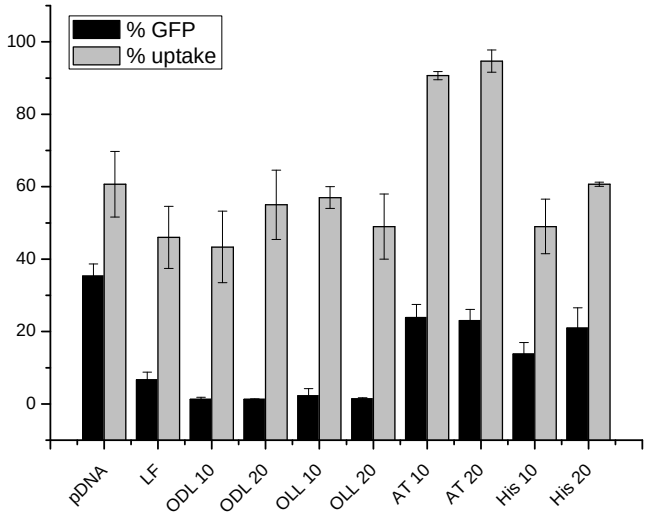


Fig. 11. Percentage of GFP positive HeLa cells (black bars) and uptake (grey bars) of pDNA/peptide complexes following electroporation. pDNA was electroporated free, or after precondensation with synthetic peptids at N/P ratio 10 or 20. As control, also pDNA complexed with lipofectamine2000® was used.

Figure 12

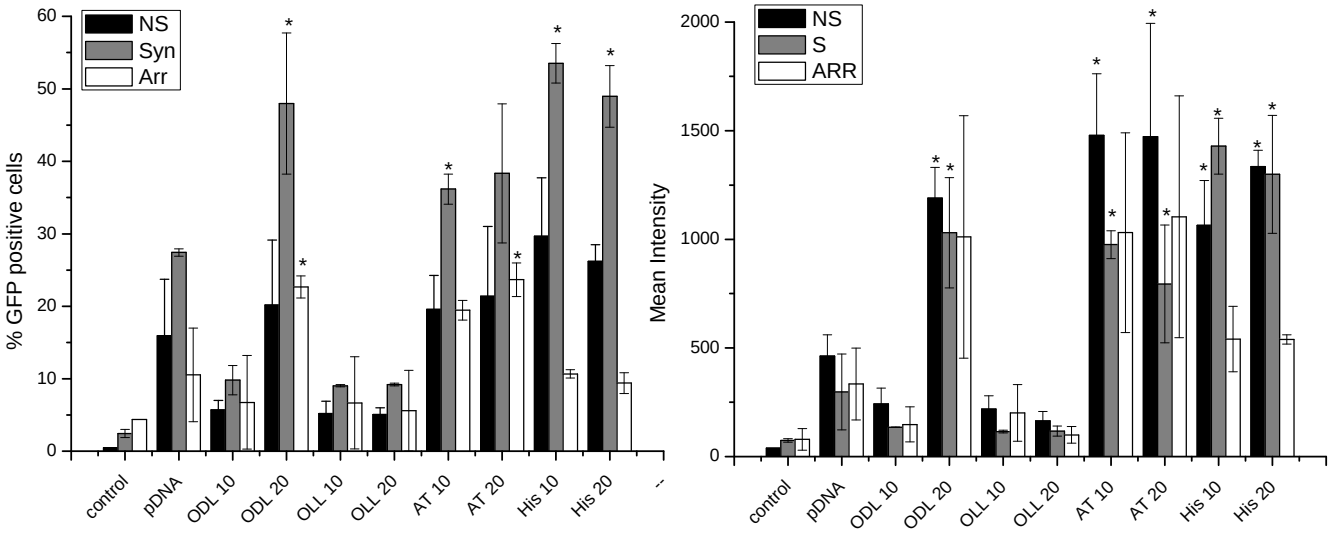
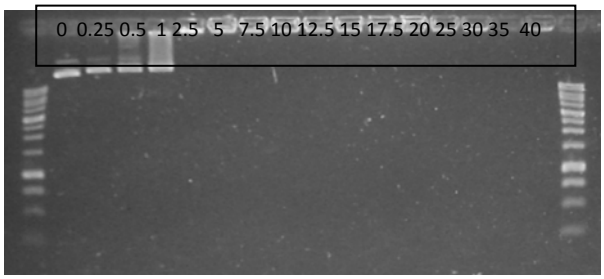


Fig. 12. Percentage of GFP positive HeLa cells (A) and mean fluorescence intensity per cell (B) for pDNA delivered by RNAimax® in free form (pDNA) or precondensed with synthetic peptides ODL, OLL, AT and his at N/P ratio 10 or 20. Transfections were performed in non-synchronized cells (black bars), synchronously dividing cells (grey bars) and arrested (non-dividing) cells (white bars). Significant differences were determined by a student t-test. * denotes a P value < 0.05.

Suppl fig 1.



Suppl Fig 1. Gel retardation assay of His in function of N/P ratio of the pDNA/peptide complexes. Each well contained 0.2 µg of pDNA. N/P ratio 0 corresponds to non-complexed pDNA.

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