Estudos biofísicos e *in vivo* de moléculas ligantes de nucleossomo

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Tese de doutorado apresentada à Universidade de Brasília como parte das exigências do Programa de Pós-Graduação em Patologia Molecular, para a obtenção do título de Doutor.

Prof. Dr. Guilherme Martins Santos

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“It's the questions we can't answer that teach us the most. They teach us how to think. If you give a man an answer, all he gains is a little fact. But give him a question and he'll look for his own answers”

(Patrick Rothfuss)

PREFACE

This work explores the results that I obtained during my PhD. Herein, I will present the results of the three projects I worked, (i) Biophyisical and *in vivo* characterization of Nucleosome Binding Peptides (NBPeps), (ii) Understanding the role of lipids on chromatin, and (iii) Investigation of peptide derived from H4 tail in phase separation. Firstly, I make a general review the many aspects of chromatin and its dynamics, followed by introduction, materials and methods, results, and discussions of the projects. (i) Part I comprises the *in vitro,* cell based and *in vivo* approach used to understand the role of NBPeps. This work suggests that chromatin can be modified by NBPeps, highlighting the importance of the nucleosome surface as a new pharmacological target and pharmacological tool. (ii) In part II, I review the current literature about lipids prevalence in the cell nucleus and the potential role of these molecules on chromatin. In this opinion type review, it is proposed new roles for lipids on chromatin. (iii) In part III, I explore findings that I obtained while investigating the role of NBPeps on the nucleosome, that suggested that one of the NBPeps, H4pep, could phase separate.

It is also presented at the end of this thesis, a paper that I was able to publish during my PhD period unrelated to my main projects, that originated during my Postgraduate education, titled Cyclophosphamide administration routine in autoimmune rheumatic diseases: a review.

RESUMO

A modulação da cromatina é feita em grande parte por Moléculas Ligantes de Nucleossomo (NBMs) que atuam interagindo com a superfície do nucleossomo, alterando a arquitetura da cromatina. Portanto, foi avaliado se Peptídeos Ligantes de Nucleossomo (NBPeps) seriam capazes de interagir com a superfície do nucleossomo diretamente e modular a cromatina, induzindo desfechos fenotípicos na célula. Para entender como NBPeps afetam a estrutura do nucleossomo, foram realizados diversos ensaios bioquímicos, que indicaram que NBPeps diferentes afetam de forma específica a estrutura do complexo, apesar de sítios de ligação semelhantes. Ensaios realizados em cultura de células mostraram que os NBPeps são captados por células Hela através de mecanismo ativo e apresentam toxicidade seletiva para células Hela, quando comparadas com células CCD10595K. Usando zebrafish (*Danio rerio)* como modelo animal demonstramos que NBPeps podem atingir o ambiente nuclear em eritrócitos de zebrafish adultos, além disso,penetram diferentes tecidos diferente da larva do peixe e induzem alterações fenotípicas em embriões, causando alterações no desenvolvimento, inibindo a produção de melanócitos, alterando a taxa de eclosão, e apresentam uma baixa taxa de letalidade. Nós concluímos que NBPeps causam desfechos fenotípicos em embriões de zebrafish, apesar de ter o sítio de ligação semelhante. Nossos resultados sugerem que NBPeps podem ter funções terapêuticas importantes.

Também foi feita uma revisão sobre os impactos de lipídios no ambiente nuclear com ênfase na modulação da cromatina, foi discutindo o possível papel do nucleossomo como reservatório de lipídeos no núcleo e seu papel na modulação da cromatina.

Além disso, foi investigado a capacidade de um NBPeps (H4pep) em induzir separação de fase *in vitro.* Foi feito ensaio de gota, mostrando que H4pep precisa de DNA para criar a separação de fase, também foi demonstrado que o complexo DNA:H4pep em altas concentrações pode alterar o estado de separação de fase, criando uma separação do tipo gel-liquida. Realizando o ensaio de resistência ao sal, foi demonstrado que H4pep separa de fase em condições fisiológicas de sal. A relevância biológica desses dados ainda não foi determinada.

Palavras-chave: Peptídeos, Nucleossomo, Cromatina, Lipídios, Separação de Fase.

ABSTRACT

The modulation of chromatin is known to be orchestrated by Nucleosome Binding Molecules (NBMs), which would act on the nucleosome surface to modulate chromatin architecture. Herein, we evaluated if Nucleosome Binding Peptides (NBPeps) would be able to occupy the nucleosome surface directly, thereby modulating chromatin status and influencing phenotypic outcomes. To understand how the nucleosome structure is affected by NBPeps, we performed biochemical assays indicating that NBPeps present differential actions on the nucleosome structure despite binding to similar target regions on the nucleosome. Cell-based assays showed that NBPeps are uptake by HeLa cells by an active mechanism and have selective toxicity for HeLa cells when compared to CCD10595K cells. Using Zebrafish models we demonstrated that NBPeps penetrated different tissues, showing specific effects on cell physiology and phenotypic outcome, altering the development of zebrafish embryos, inhibiting the development of melanocytes, changing hatching patterns and inducing death at a small rate. Moreover, analysis in adult zebrafish showed that NBPeps can reach the nuclear environment of erythrocytes *in vivo*. We concluded that NBPeps present specific phenotypic outcomes in zebrafish embryos despite having a similar binding sites. Taken together, our data suggests that NBPeps might have important therapeutic implications.

Herein, we also reviewed the impacts of lipids on the nuclear environment, discussing the potential role of nucleosome as a reservoir of lipids in the nucleus and, also emphasizing the lipids as a modulator of chromatin architecture.

Finally, we investigated the capacity of a NBPep (H4pep) in creating phase separation *in vitro*. Droplet assay shows that H4pep requires DNA to crate phase separation, also that the complex DNA:H4pep at high concentrations can change phase creating a gel-liquid phase separation. Salt resistance assay shows that H4pep creates phase separation at a physiological concentration of NaCl. The relevance biological relevance of this data is yet to be determined.

Keywords: Peptides, Nucleosome, Chromatin, Lipids, Phase Separation.

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LIST OF ABBREVIATIONS

ALS	Amyotrophic Lateral Sclerosis
ASH2L	Histone lysine methyltransferase complex subunit
ATP	Adenosine Triphosphate
AUC	Analytical Ultra Centrifugation
CCAN	Constitutive Centromere Associated Network
CD	Circular Dichroism
CENP-A	Centromere protein A
CENP-C	Centromere protein C
CENP-N	Centromere Protein N
Chd1	Chromodomain-helicase-DNA-binding protein 1
cLD	cytoplasmatic Lipids Droplets
COMPASS	Complex of Proteins Associated with Set1
Cryo-EM	Cryogenic Electron Microscopy
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DNMT	DNA methyltransferase
Dot1L	Disruptor of Telomeric Silencing 1-Like
DUB	Deubiquitinating enzymes
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic Acid
FACT	Facilitates Chromatin Transcription
FET	Fish Embryo Toxicity
FTD	Frontotemporal Dementia
GCN4	General control protein GCN4
GMIP1	Genetic Modified Inducible Peptide 1
HAT	Histone Acetyl Transferase
HDAC	Histone deacetylases
HDACi	Histone deacetylases inhibitor
HGPS	Hutchison-Gilford Progeria Syndrome
HMGN2	High Mobility Group Nucleosomal 2
HO	Histone Octamers
HP1α	Heterochromatin protein 1 alpha
HSQC	Heteronuclear Single Quantum Coherence
IDRs	Intrinsically disordered regions
IL-33	Interleukin-33

iMEF	immortalized murine embryonic fibroblasts
INO80	Inositol-requiring 80
ISWI	Imitation SWI
KSHV	Kaposi's Sarcoma Herpesvirus
LANA	Latency Associated Nuclear Antigen
LC-MS	Liquid Chromatography Mass Spectotmetry
LEDGF	Lens epithelium–derived growth factor p75 splice variant
LLPS	Liquid Liquid Phase Separation
MBM	Minimum Binding Motif
MLL1	Mixed linage leukemia
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBP	Nucleosome Binding Proteins
NBPeps	Nucleosome Binding Peptides
nLD	nuclear Lipids Droplets
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
OTC4	Octamer-binding transcription factor 4
PEG	Polyethylene Glycol
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PTM	Post Translational Modification
RbBP5	Retinoblastoma-binding protein 5
RCC1	Regulator of Chromosome Condensation 1
RNA	Ribonucleic acid
RNAPII	RNA polymerase II
RSC	Remodeling the Structure of Chromatin)
SAGA	Spt-Ada-Gcn5 acetyltransferase
SAXS	Small Angle X-ray Scattering
Set2	SET domain-containing protein 2
SG	Stress Granules
SIR 3	Silent Information Regulator 3
Snf2	Transcription regulatory protein SNF2
Snf5	SWI/SNF chromatin-remodeling complex subunit SNF5
SOX11	SRY-box transcription factor 11
SOX2	Sex determining region Y
SWI/SNF	Switch/Sucrose Non-Fermentable
TAMRA	5-(and-6)-Carboxytetramethylrhodamine
TBE	Tris Boric Acid EDTA

CHAPTER I

1. GENERAL INTRODUCTION

Even before the discovery of the DNA in 1868 (reviewed in (Dahm 2005) and further with its structure determination by Watson and Crick in 1953 (Watson and Crick 1953), the life code was of great interest for scientists, to the point today several fields of science are dedicated to understanding it, making a crucial knowledge field on modern life. Consequential progress has been made and our understanding of how the DNA works inside a cell has improved significantly. For instance, it is known that in eukaryotic cells, the DNA is tightly packed inside the nucleus, and this packing forms a hierarchical structure that is finely regulated.

1.1. The nucleosome and the chromatin

The nucleosome is the first structure in the hierarchy that organizes the DNA inside the cell nucleus. It is composed of DNA and a disk-shaped octamer of proteins known as histones (H2A, H2A, H3, and H4), that works as a scaffold for the DNA to wrap around making 1.7 turns, as seen in Figure 1 (Luger, Mader et al. 1997).

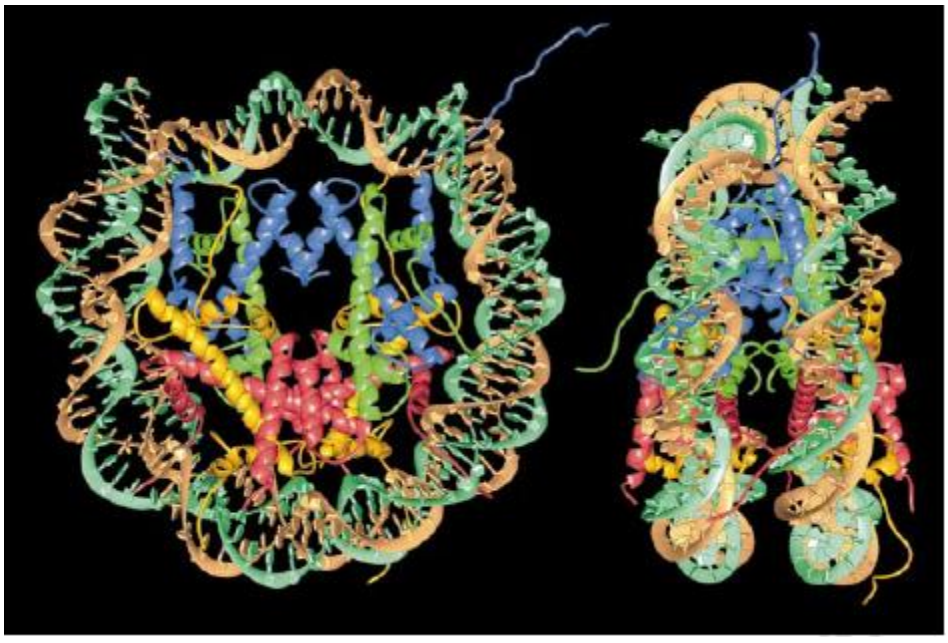

Figure 1: Side and front view of the first high-resolution crystal structure of the nucleosome. In brown and turquoise, 146 bp of DNA wrapping around the octamer of canonical histones; H2A in yellow; H2B in red; H3 in blue and H4 in green. Adapted from (Luger, Mader et al. 1997)

This unit is repeated several times, connected by linker DNA, and forming the second hierarchical structure of DNA packing in the nucleus, the chromatin (Figure 2). This structure can assume different conformations, the first is said to be the beads-on-a-string, or euchromatin. Internucleosomal interactions can occur creating the compacted chromatin, or heterochromatin, also known as the 30 nm fiber. These interactions induce more compacted states going through chromonema, chromatid and finally, during mitosis the chromatin can compact even further forming chromosomes (reviewed in (Bickmore and van Steensel 2013) (Woodcock 2006).

The compaction and relaxation of chromatin are vital for the regulation of a myriad of cellular processes, given that when the chromatin is compacted, the access of the basal transcriptional machinery is blocked, silencing gene expression; when is relaxed, DNA is exposed, allowing the basal transcriptional machinery to interact with the genetic material. For these reasons the chromatin is considered to be the first major transcriptional barrier (Bintu, Ishibashi et al. 2012).

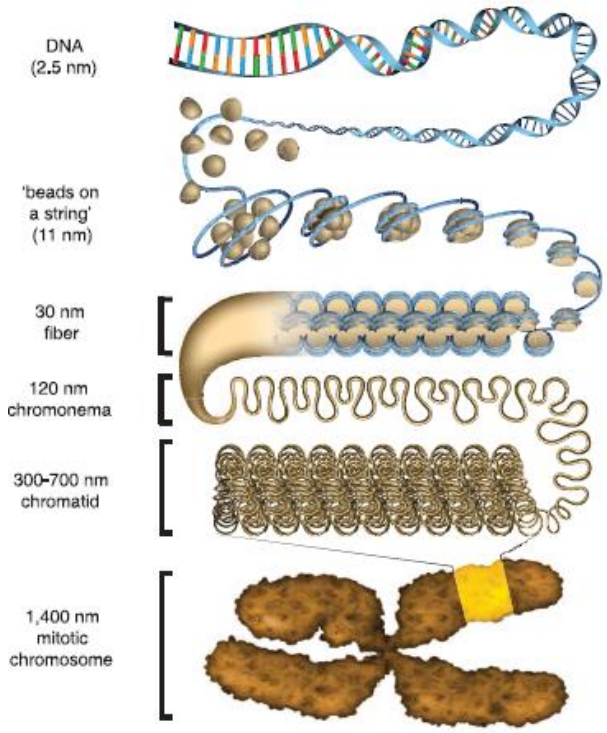

Figure 2: Chromatin compaction schematics. Going from free DNA through the mitotic chromosome and their respective sizes in thickness. Adapted from (Ou, Phan et al. 2017)

1.2. Chromatin Dynamics

The compaction of chromatin, is a major component for gene expression regulation, thus there are a plethora of manners that it can be regulated. The most well-characterized aspect of the compaction and relaxation phenomenon is the histone H4 tail interaction, in this case, internucleosomal interactions can occur, mediated by the N-terminus region of the H4 histone that binds to an acidic region of the neighboring nucleosome, known as acidic patch (Luger, Mader et al. 1997). The histone H4 tail was confirmed by Dorigo *et al.* to be the only histone tail to be necessary to induce compaction in the chromatin (Dorigo, Schalch et al. 2003). But this interaction can be modified by Post Translational Modifications (PTMs), that are mediated by enzymes such as Histone Acetyl Transferase (HAT), which deposits an acetyl group in the lysine 16 of the H4 histone, neutralizing the net charge of this amino acid, thus preventing the interaction with the acidic patch of the neighbor nucleosome, hence inducing the relaxed state in the chromatin. The

enzyme Histone Deacetylase (HDAC) can undo this process, promoting the compaction of chromatin (Marmorstein and Roth 2001). Furthermore, HDAC inhibitors (HDACi) are a class of drugs, that are used to treat a myriad of diseases, from neurological disorders, such as T-cell lymphoma, multiple myeloma, epilepsy, bipolar disorders and migraine (Eckschlager, Plch et al. 2017), and recently has been proposed as a potential treatment for the COVID-19 infection (Gordon, Jang et al. 2020).

Another factors that induce chromatin compaction is the presence of mono or divalent cations. Using AUC (Analytical Ultra Centrifugation) technique, Korolev and collaborators showed that the higher the cation charge, fewer ions were necessary to induce condensation of the chromatin, with the best being Mg^{2+}. This happens due to neutralization of residual charges in the DNA, facilitating the internucleosomal interaction (Lundberg, Berezhnoy et al. 2010).

The histone H1 is a non-canonical histone that is not part of the histone octamer and binds to the chromatin. It was thought to bind to the dyad region of the nucleosome (entry and exit points of the DNA in the nucleosome), acting as a clamp, inducing chromatin compaction (Robinson, An et al. 2008, Song, Chen et al. 2014). However, it was revealed a new mechanism of compaction mediated by this histone. Using SAXS (Small Angle X-ray Scattering) and fluorescent microscopy techniques, it was showed that this protein induces a phase separation when bounded to the chromatin, potentially shielding the separated phase from other molecules and inducing compaction, as seen in Figure 3 (Larson, Elnatan et al. 2017, Strom, Emelyanov et al. 2017).

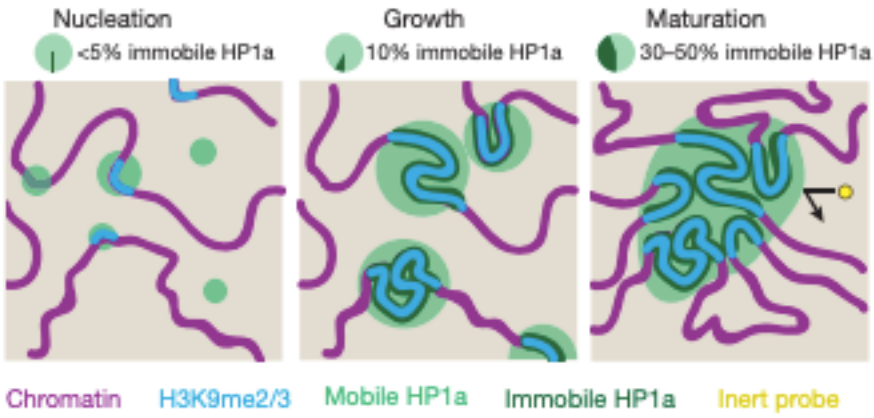

Figure 3: Chromatin phase separation and compaction. Histone HP1a recognize PTMs in histones and binds inducing a compacted and phase separation in the chromatin, preventing other molecules

to interact with it. Adapted from (Strom, Emelyanov et al. 2017).

Further studies in the role of phase separation and chromatin dynamics revealed that in fact, reconstituted chromatin undergo histone tail-driven liquid-liquid phase separation, also that linker DNA lengths and histone H1 play an important role in the formation of droplets, impacting over some relevant characteristics. Furthermore, it was revealed that acetylation by p300, antagonizes the formation of phase separation, indeed, in the presence of highly acetylated chromatin, the formation of a different phase-separated environment that is immiscible with the non-acetylated chromatin happens, Figure 4 shows a model of how chromatin dynamics are dictated by phase separation. These recent studies are revealing a whole new mechanics for chromatin regulation that are highly dependent on the phase separation phenomenon.

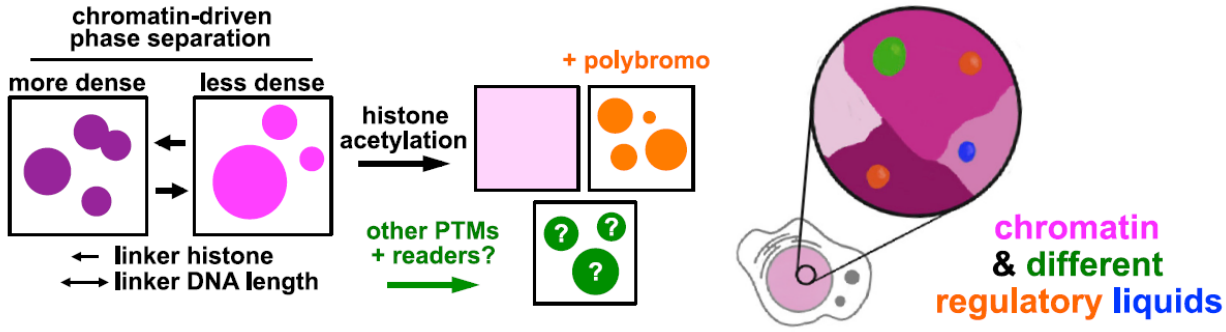

Figure 4: Chromatin regulation by phase separation. A model for chromatin dynamics regulated by distinct membranelles subdomains. Adapted from (Gibson, Doolittle et al. 2019)

2. PART I - NBPeps

2.1. Nucleosome Binding Proteins

One of the main forms of chromatin remodeling and regulation is mediated by NBPs (Nucleosome Binding Proteins). Several of these proteins have been identified, some with the detailed mechanism of action and atomic levels resolution of interaction with the nucleosome surface, here I summarized some NBPeps that have been identified interacting with the nucleosome, I briefly describe how it interacts, and what function of the NBPs over the chromatin.

In 2010, Song Tan and collaborators obtained the first structure of a full length protein bounded to the nucleosome solved by x-ray cystography. They were able to obtain the protein

RCC1 (Regulator of Chromosome Condensation 1) with the structure with the resolution at 2.9 Å, providing an atomic overview of how this interaction happens. They observed that the RCC1 interacts in two distinct regions of the nucleosome, one in the acidic patch located in the histones H2A and H2B and other making contact with the nucleosomal DNA (Makde, England et al. 2010).

Four years later the same group determined the structure of the domain responsible for ubiquitination in PRC1 (Polycomb Repressive Complex 1). They showed that this protein binds mostly to the nucleosomal DNA and a small negative zone, near the acidic patch (McGinty, Henrici et al. 2014).

Another major contribution to the field was the structure of the peptide Latency Associated Nuclear Antigen (LANA) present in infections by Kaposi's Sarcoma Herpesvirus (KSHV). This peptide is 23 amino acids long and is associated with the latency period of the disease (Ballestas, Chatis et al. 1999). At the resolution of 2.9 Å it was possible to visualize that this peptide binds to the acidic patch of the nucleosome in a hairpin manner (Barbera, Chodaparambil et al. 2006).

To acquire good structural data in large macromolecular structures like the nucleosome, is always challenging. Frequently, large molecules cannot be crystallized or analyzed by NMR (Nuclear Magnetic Resonance) (Nogales and Scheres 2015). In 1997 the first protein structure was solved using Cryo-EM, which opened a new era for the resolution of large macromolecular complexes (Böttcher, Wynne et al. 1997). Kurumizaka and collaborators accomplished the astounding feature of determining how RNAPII (RNA polymerase II) interacts with the nucleosome and even how it can surpass the complex, using Cryo-EM (Kujirai, Ehara et al. 2018). They showed that RNAPII pause at specific regions of the DNA and that RNAPII gradually tears DNA from the histone surface while preserving the histone octamer.

An important discovery about how viruses can integrate their genome into the host was elucidated by Costa and colleagues, they used foamy virus intasome engaged with a nucleosome, analysis with Cryo-EM and Förster resonance energy transfer measurements to show that the retroviral integrase twist and slide nucleosomal DNA by approximately two base pairs, lifting from histones H2A/H2B to allow engage with the intasome (Wilson, Renault et al. 2019).

In 2011 a peptide from the SIR3 (Silent Information Regulator 3) was co-crystalized with the nucleosome, and differently from all others proteins or peptides that have been observed to that date, did not interact with the acidic patch region or surrounding residues, but mostly with histone H3 (Armache, Garlick et al. 2011).

The SAGA (Spt-Ada-Gcn5 acetyltransferase) contains a DUB (Deubiquitinating enzymes) module, it is responsible for the regulation and deubequitintion of H2B, involved in a myriad of gene regulation processes (Bonnet, Devys et al. 2014). In 2016 Wolberger and collaborators, using X-ray crystallography, revealed the mode of interaction of this complex with the nucleosome. They showed that interactions occur mostly at the acidic patch and are involved in the different stages of histones disassembling (Morgan, Haj-Yahya et al. 2016).

The protein CENP-C (Centromere protein C) has its function in the assembly of kinetochore proteins, mitosis, and the segregation of chromosomes. In 2013 the mode of interaction between a peptide from this protein and the nucleosome was determined using Nuclear Magnetic Resonance (NMR) and X-ray crystallography. They observed that the CENP-C peptide interacts with the N-terminus of a H3 histone variant, known as CENP-A (Centromere protein A) that further binds to the acidic patch (Kato, Jiang et al. 2013). More insights about how kinetochore works was revealed by Bradford and collaborators, they showed that nucleosome containing CENP-A bound to CCAN (Constitutive Centromere Associated Network) from Sac*charomyces cerevisiae*, indicating the mechanism of CENP-A nucleosome recognition by CCAN and its role as a platform for assembly of the outer kinetochore to link centromeres in the mitotic spindle formation during chromosome segregation (Yan, Yang et al. 2019). Using the same technique, Musacchio and collaborators investigated the mode of interaction of the protein Centromere Protein N (CENP-N) with the non-canonical histone variant CENP-A, previously mentioned. They revealed that CENP-N interacts largely with 15bp of nucleosomal DNA, preventing further NBPs to bind to the region and also a new binding motif identified in CENP-A (Pentakota, Zhou et al. 2017).

In a very elegant work, Ingen and collaborators determined the binding epitopes of a peptide from the High Mobility Group Nucleosomal 2 (HMGN2) using methyl-based NMR analysis. They showed that the interaction was similar to the RCC1, with one binding site interacting with the acidic patch, and other to the DNA (Kato, van Ingen et al. 2011).

The Interleukin-33 (IL-33) is a protein that can act as a cytokine, when in the extracellular environment, and as a nuclear receptor when intracellular (Pichery, Mirey et al. 2012, Fu, Hung et al. 2016). A peptide from this protein was also observed by NMR to bind to the nucleosome in a similar manner to LANA, making contacts exclusively with the acidic patch (Roussel, Erard et al. 2008).

The LEDGF (Lens epithelium–derived growth factor p75 splice variant) is a NBP with antiapoptotic properties known to direct human immunodeficiency virus into active transcription units (Daugaard, Baude et al. 2012). In 2020, the domains PWWP (proline, tryptophan, tryptophan, proline) was obtained with methylated nucleosome, showing the cooperative interaction between the multivalent binding of the reader domains to the methylated histone tail from H3 and to both gyres of nucleosomal DNA (Wang, Farnung et al. 2020).

There are several histone chaperons with important function into chromatin remodeling and nucleosome assembly/disassembly, FACT (Facilitates Chromatin Transcription) is one of these chaperones, playing important roles during gene transcription, DNA replication and, DNA repair. In 2019, Luger and collaborators, using Cryo-EM and biochemical assays revealed the mechanism by which FACT operates, showing that FACT engages with nucleosomal DNA and several histones with PTMs, demonstrating that a complex of FACT-H2A/H2B is formed, which can interact with H3/H4, allowing the assembly/disassembly process (Liu, Zhou et al. 2020).

Several NBPs require specifics PTMs to properly interacts with the nucleosome, the protein Dot1L (Disruptor of Telomeric Silencing 1-Like) requires histone a monoubiquitination in the H2B lysine 120 (H2BK120Ub) to be able to methylate the lysine 79 of histone H3 (H3K79m), showing a histone crosstalk phenomenon. Using cryo-EM, the group led by Valencia-Sánchez provided structural and functional data as well as the correlation between aberrant H3K79m and leukemia, suggesting the modulation of Dot1L as a therapeutic target for this disease (Valencia-Sanchez, De Ioannes et al. 2019).

Mutations in the Set2 (SET domain-containing protein 2) enzymes are related to cancer progression, these methyltransferase enzymes, recognizes H3K36me and H2B-Ub nucleosome, in 2019 their mode of interaction was determined by Cryo-EM, showing mostly contacts with histone H3, H2A C-terminal and unwrapped DNA, Intriguingly it was revealed that the interfaces that can be targeted with small molecules for the future development of cancer therapies (Bilokapic and Halic 2019).

Acting in the same region, the COMPASS (Complex of Proteins Associated with Set1) complex, is formed by six proteins with important methyltransferase activity, in 2019, a group led by Wolberg and colleagues solved the structure of COMPASS bound to ubiquitinated nucleosome using Cryo-EM, their work revealed a long-standing mystery of how H2B-Ub is recognized by COMPASS and provided the first trans-nucleosome histone reveled crosstalk mechanism

(Worden, Zhang et al. 2020).

Still regarding the roles of NBPs in methylation of histones, the structure of the complex MLL1 (Mixed linage leukemia) with the nucleosome was obtained by Cryo-EM, showing that the subunit RbBP5 (Retinoblastoma-binding protein 5) and ASH2L (histone lysine methyltransferase complex subunit) make large interactions with the nucleosome dyad, nucleosomal DNA and the N-terminus tail from histone H4, shedding light on how the MLL1 complex engages chromatin and the tri-methylation activity of the complex (Park, Ayoub et al. 2019).

ATP dependent remodeling of the chromatin is made by a diverse family of proteins that have an ATP-ase domain. Using Cryo-EM, the interaction motifs of several of these proteins have been identified. Chd1 (Chromodomain-helicase-DNA-binding protein 1) is part of this important family of proteins and works as an organizing nucleosome over codding regions (Ocampo, Chereji et al. 2016). The binding mode of this protein to the nucleosome was shown to be mostly with linker DNA and histone H3, induce unraveling of DNA and reorientation of H3 tail (Sundaramoorthy, Hughes et al. 2018).

The INO80 (inositol-requiring 80), a chromatin remodeler, that is ATP dependent, is composed by multi-subunits. It was previously thought the H4 tail played a major role in regulating some of its units (van Attikum and Gasser 2005). However, Zhang and collaborators, using Cryo-EM, showed a new mode of binding involving nucleosomal DNA and H3 as well as the fact that the H3 tail instead is responsible for this regulation (Ayala, Willhoft et al. 2018).

The SWI/SNF (Switch/Sucrose Non-Fermentable) is a chromatin remodeling complex and has important roles in transcription and DNA-damage repairs, this complex can hydrolase ATP and evict or slide histone octamers, creating exposed DNA regions for other proteins, such as transcriptional factors. In 2020 He and collaborators, using Cryo-EM, were able to obtain near-atomic resolution of this complex from *Saccharomyces cerevisiae* bound to the nucleosome, giving valuable insights about how this complex works. They showed the protein Snf5 (SWI/SNF chromatin-remodeling complex subunit SNF5) interacts with the acidic patch, functioning as an anchor for the whole complex during active DNA translocation (Han, Reyes et al. 2020). Furthermore, domains of this complex, such as Snf2 (Transcription regulatory protein SNF2) and ISWI (imitation SWI) where elucidated a year before the whole complex resolution, showing interactions with nucleosomal DNA and strikingly similar binding epitopes, suggesting a conserved mechanism for chromatin remodeling (Li, Xia et al. 2019, Yan, Wu et al. 2019). In a

similar manner, the RSC (Remodeling the Structure of Chromatin) from e *Saccharomyces cerevisiae*, which is part of the SWI/SNF family was elucidated by Cryo-EM in 2019 by Nogales and collaborators, their findings shed light on the structural insights into the conserved assembly process for members of SWI/SNF family of remodelers, showing how RSC selects, engages and remodel nucleosomes (Patel, Moore et al. 2019).

Recently the structure of two pioneers transcriptional factors SOX2 (Sex determining region Y) and SOX11 (SRY-box transcription factor 11) was solved using Cryo-EM, Cramer and collaborators showed that the transcriptional factors can bind and distort superhelical DNA at the position +2 , facilitating the detachment of terminal nucleosomal DNA from the histone octamer. Furthermore, upon SOX-factor binding, can lead to a repositioning of the N-terminal tail from histone H4, including the lysine 16, which has an important role in the regulation of chromatin compaction via the interaction with the acidic patch from the neighbor nucleosome, suggesting that SOX2 and SOX11 prevent the formation of higher-order chromatin, thereby facilitating nucleosome remodeling and subsequent transcription (Dodonova, Zhu et al. 2020).

With the exception of the ATP dependent remodeling family of proteins identified interacting with the nucleosome, RNAPII, and the SOX family, the majority of NBPs focus on the acidic patch as their binding site, as seen in Figure 5.

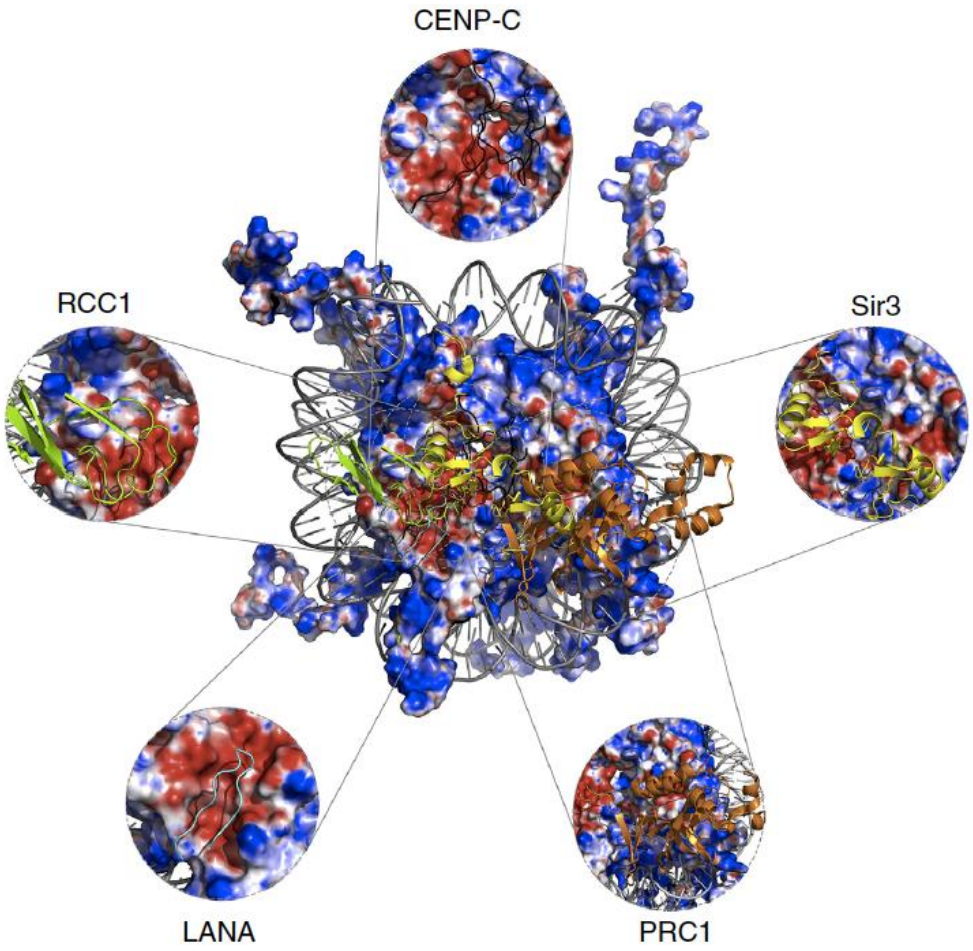

Figure 5: Acidic patch is a docking hub for NBPs. Overall charge view of the nucleosome, in blue positive and red negative. The acidic patch is highlighted and NBPs are overlaid. Adapted from (Cabral, Machado et al. 2016)

2.2. The acidic patch and NBPeps

As addressed in the previous section, the acidic patch can work as a docking hub for several NBPs, thus having an important role in gene regulation. This idiosyncratic region in the nucleosome surface is composed of 8 acidic amino acids between the histones H2A and H2B (E56, E61, E64, D90, E91, E92 of H2A and E102, E110 of H2B).

In 2014, in an attempt to displace LANA from the acidic patch and treat the latency aspect associated with KSVH, a group led by Keye, screened over 350,000 small molecules and all failed to do it. The authors concluded with the suggestion that more complex molecules, such as peptides,

might be a better option to displace the LANA from the nucleosome (Beauchemin, Moerke et al. 2014).

Due to the regulatory nature of the acidic patch, and the findings of Beachemin et al., Dr. Santos suggested that the nucleosome surface, and in specific the acidic patch, could be a potential pharmacological target using peptides for it (Silva, de Oliveira et al. 2015). In Dr. Santos's paper, it is theorized that the binding of Nucleosome Binding Peptides (NBPeps) can induce specific outcomes in the chromatin and modulate cell function, as seen in Figure 6. In a similar manner of other epidrugs, NBPeps would modulate the chromatin architecture in a non-specific way. However, in this case changes would be direct to the chromatin, and not mediating chromatin remodeler enzymes. Furthermore, occupying the binding site for several NBPs can prevent the binding of several of these proteins, thus modulating chromatin architecture.

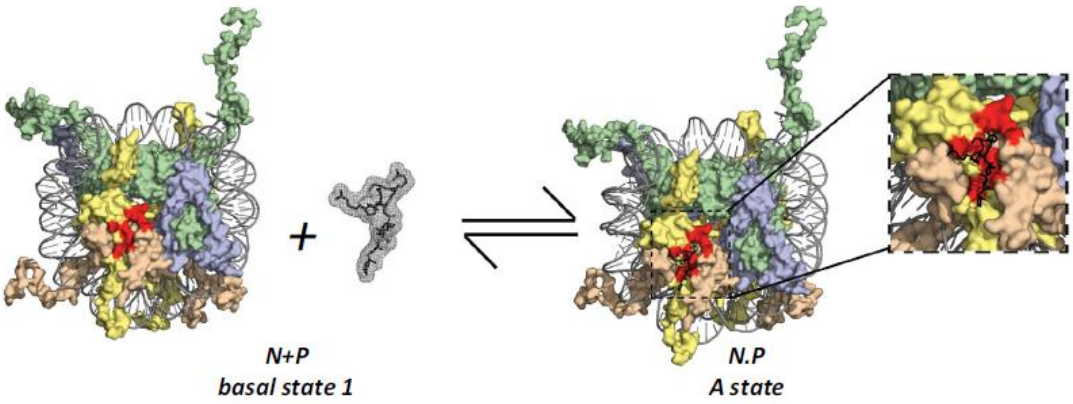

Figure 6: Kinetics of drug receptor for the nucleosome. The nucleosome and LANA peptide from Protein Data Bank (PDB) 1ZLA) are represented. H3 in green; H4 in blue; H2A in yellow; H2B in wheat; DNA in gray; acidic patch in red. The dynamic equilibrium between the nucleosome and NBPep, resulting in two states, bound or unbound to the nucleosome. Adapted from (Silva, de Oliveira et al. 2015).

In a novel work led by Dr. Luger, they developed binuclear ruthenium compounds that bind to the acidic patch, inducing aberrant chromatin condensation and alterations in the cell's cycle, with potential applications in drug development and as tools for chromatin research (Davey, Adhireksan et al. 2017). It demonstrated that chromatin can in fact be modulated by exogenous

molecules.

For the development of this work, four NBPeps were used, with GMIP1(Genetic Modified Inducible Peptide 1) being designed *in silico* and LANA, HMGN2pep, and H4pep based on the MBM (Minimum Binding Motif) of these proteins that were already known to bind to the nucleosome, generating new peptides. Table 1 shows the sequence and mode of creation of these peptides.

Table 1: List of all NBPeps used in this work, the amino acid sequence, and method of resolution alone and in complex with the nucleosome.

NBPep	Sequence	Atomic Coordinates	
		Single	**Complex**
H4pep	RGKGGKGLGKGGAKRHRKVLR	online server (I-tasser) based on (Yang and Arya 2011)	Molecular Docking based on (Yang and Arya 2011)
HMGN2pep	DEPQRRSARLSAKPAPPKPEPKPKK	NMR (Kato, van Ingen et al. 2011)	NMR (Kato, van Ingen et al. 2011)
LANA	PGMRLRSGRSTGAP	X-ray (PDB code 1ZLA (Barbera, Chodaparambil et al. 2006))	X-ray cristallography (PDB code 1ZLA (Barbera, Chodaparambil et al. 2006)
GMIP1	RTIIAAALSERSISGEGRR	Kvfinder (Makde, England et al. 2010) (Oliveira,	Molecular Docking

	Ferraz et al. 2014)

The design of GMIP1 was based on the mode of interaction of RCC1 using the software KVFinder (Oliveira, Ferraz et al. 2014). It was simulated the mode of interaction of four proteins, RCC1 having the best fit (Teles, Fernandes et al. 2020). The binding mode of this protein, as previously stated, consists of one-part binding to the acidic patch and other to the DNA. The MBM of these two regions were connected by a three-alanine bridge, as seen in Figure 7.

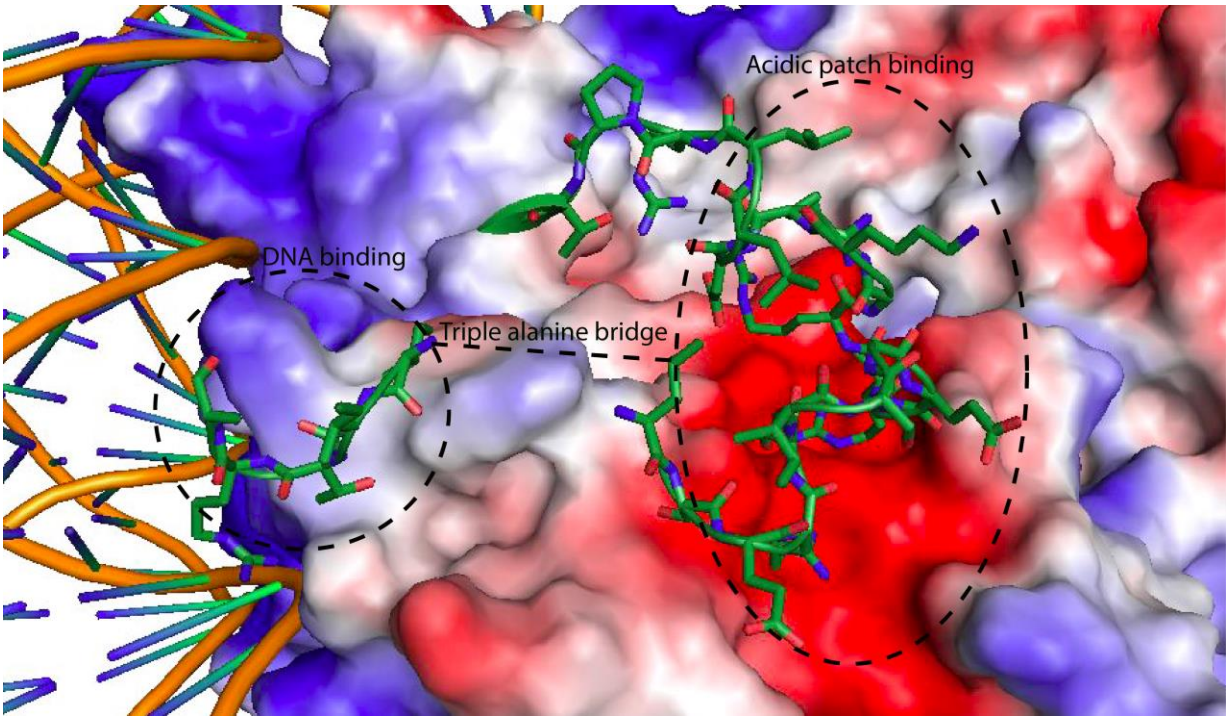

Figure 7: Mode of binding of RCC1 and GMIP1 design. The MBM of RCC1 with the two regions of interaction with the nucleosome highlighted and the triple alanine bridge region connecting the two distinct regions. Images extracted and modeled using PDB (3MVD) from (Makde, England et al. 2010) and PyMol software.

In this work, I performed *in vitro,* cell-based assays, and *in vivo* assays in order to very if NBPeps (Nucleosome Binding Peptides) designed by Dr. Santos' group have therapeutic potential

purposes and if targeting the nucleosome in order to module cell phenotypes is a feasible option to module cell phsyiology. The atomistic characterization of how NBPeps would affect the nucleosome was done by Fernandes. V, using *in silico* strategies at LBTC.

3. AIMS PART I (NBPeps)

3.1. PRIMARY AIM

This work's goal is to understand how NBPeps that present distinct nucleosome binding sites affect the nucleosome and chromatin structure, thereby modulating chromatin status and influencing phenotypic outcomes.

3.2. SECONDARY AIMS

- Verify NBPeps binding to the nucleosome *in vitro;*
- Verify cellular uptake of NBPeps;
- Verify the toxicity of NBPeps in different cell lineages;
- Verify the toxicity of NBPeps in zebrafish embryos;
- Verify the distribution of NBPeps in tissues of zebrafish larvae;
- Verify if NBPeps can reach the nucleus of cells *in vivo.*

4. METHODS

NBPeps: All Peptides were bought from Biomatik with 95%> purity and diluted in MiliQ H_2O. Fluorescent peptides were bought with TAMRA -(559/583nm) in the N-terminus. The concentration was determined by spectrophotometric method as described in (Murphy and Kies 1960). All peptides are described in Table 1.

***In vitro* nucleosome reconstitution**: Histone octamers (HO) were purified from chicken erythrocyte nuclei as described in Huynh, V. A. T., P. J. J. Robinson, and D. Rhodes, 2005. 601 DNA Widom with 167 base pairs (bp) was used to reconstitute mononucleosomes, using the slow salt dialysis method as described in (Huynh, Robinson et al. 2005).
The analyses of the reconstitution were verified by electrophoresis in native bis-acrylamide gels (6%).

Mononucleosome precipitation: Freshly reconstituted mononucleosomes (115nM mononucleosome, Tris 10mM pH 7.4, EDTA 1.5mM NaCl 15mM) were incubated with the specified concentration of NBPeps for 30 minutes at room temperature. The samples were centrifuged (Sigma centrifuge-2K15) at 15493 x g for 20 minutes at 25 °C. The supernatant was transferred to another microcentrifuge tube and the pellet was resuspended in the same buffer as the mononucleosome. The samples were analyzed by electrophoresis in native 6% bis-acrylamide gel carried out with 0.5× TBE buffer at 15 mA. Densitometry was performed using ImageJ (National Institute of Health, Bethesda, MD, USA) version 1.49.

DNA binding assay: Widom 601 DNA fragments containing 167bp (30nM DNA, 10mM Tris pH 7.4, 135mM NaCl) were incubated with specified concentrations of GMIP1 for 2 hours at 37 ◦C and 100 RPM. The analysis was done in 0.8% agarose gel in TBE 0.5X. Samples were loaded with 30% glycerol, to avoid interaction caused by phenol blue and GMIP1.

Nucleosome binding assay: Freshly reconstituted mononucleosomes (115nM mononucleosome, Tris 10mM pH 7.4, EDTA 1.5mM NaCl 15mM) were incubated with the specified concentration of fluorescent NBPeps for 120 minutes at room temperature. Then samples were analyzed by electrophoresis in native 6% bis-acrylamide gel carried out with 0.5× TBE buffer at 15 mA. Gels

were analyzed using Amersham Imager 600 (GE) with the RGB laser kit detection for 520nm, to visualize the peptide, following incubation in ethidium bromide bath and analyzed with UV for ethidium bromide detection. For Kd determination, band densitometry was performed in the gel reveled with 520nm laser, using ImageJ (National Institute of Health, Bethesda, MD, USA) version 1.49, followed by analysis in Prism 6 Graphpad software using Binding - saturation binding to total and non-specific template.

MTT: For MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays, 8000 Hela cells or ccd10595k cells were plated in 96-well culture plates and maintained at 37◦C and 5% CO_2 in DMEM medium with 10% fetal bovine serum, penicillin (100U/mL) and streptomycin (100ug/mL) for 24 hours. Next, wells were washed 3 times with PBS 1X and filled with 100uL of DMEM medium as described above containing the specified amount of NBPeps and incubated for 24 hours in the same conditions. The MTT at 5mg/mL was added to the wells (10uL) and incubated for 4 hours at 37◦C and 5% CO_2. The wells were drained, and the formazan crystals were solubilized in 100uL of acidic isopropanol solution (52uL of HCl 37% to 12 mL of isopropanol) and agitated for 30 minutes at room temperature. Absorbance at 570nm was determined with a plate spectrophotometer (DTX 800 Multimode Detector - Beckman Coulter) at 570 nm.

Flow cytometry: 70.000 Hela cells were plated in 12-wells culture plates for 16 hours and maintained at 37◦C and 5% CO_2 in DMEM medium with 10% fetal bovine serum, penicillin (100U/mL) and streptomycin (100ug/mL). Prior to treatment with NBPeps, cells were incubated for 1 hour at 37 or 4 ◦C. Next, cells were washed with 1X PBS and filled with DMEM medium with the specified amount of fluorescent NBPeps and incubated at 37 or 4 ◦C for 1 or 3 hours. Wells were washed three times with ice cold 1X PBS and filled with 500uL 1X PBS, cells were harvested with a cell scraper and analyzed by flow cytometry on FACSCalibur (BD biosciences). Hela cells were gated to isolate the main population of living cells from cell debris. Data analysis was done using flowjo 8.7 software.

Zebrafish husbandry and embryo collection: Zebrafish *(Danio rerio)* were raised in an aquatic facility (ZebTec - Tecniplast, Italy) with a photoperiod cycle of 12:12 h (light:dark) at the University of Brasilia (Brazil). The water parameters were: temperature was maintained at 27.0 ±

1 °C, conductivity at 650 ± 100 µS/cm, pH at 7.0 ± 0.5 and dissolved oxygen≥95% saturation. Zebrafish embryos were collected immediately after natural mating, rinsed in water, and checked under a stereomicroscope (Stereoscopic Zoom Microscope – Stemi 2000, Zeiss, Germany). The unfertilized eggs and those showing cleavage irregularities or injuries were discarded (OECD 2013).

Fish embryo toxicity (FET): FET was adapted from Morash et al (Morash, Douglas et al. 2011). Briefly, Zebrafish embryos at 4, 28 and 52 hours post fertilization (hpf) were used to evaluate the toxicity of NBPeps in 96-well plates. Each peptide was tested at 0.1, 1, 10 and 100uM in 100uL of water from the aquarium system; pH in all conditions was tested using pH strips (92120 – MACHEREY-NAGEL). Embryos were stored at 27 ◦C with 14 hours light 10 hours dark cycle and evaluated Stemi 508 (Carl Zeiss) microscope with 1 and 24 hours of treatment. Embryos were assessed for pigmentation, development, hatching and lethality. 10 embryos were used for each condition, if the control group showed any alteration, the plate was discarded, alterations >10% were considered significant and were documented using Axiocam Erc 5s (Carl Zeiss) and ZEN software (Carl Zeiss).

Fluorescence fish embryo: Zebrafish larvae with 80 hpf were incubated with fluorescent NBPeps with specified concentration for 3 hours in 100uL in a 96-plate, larvae were washed 3 times in 100mL to remove the excess of NBPep, imaging was done using Axioskop 2 (Carl Zeiss) with HBO 100 lamps, Axiocam Erc 5s (Carl Zeiss) and ZEN software (Carl Zeiss) with appropriate laser filter for TAMRA (filter 4).

Fluorescence blood smear: Adults Zebrafish at 2 years old were injected in the abdomen with 50uL, 1mM of fluorescent NBPeps, and kept protected from light at 27 ◦C for 18 hours. Blood was extracted from the fins using a pipet tip and heparin 250 IU to make the blood smear in a microscope slide. Images were acquired with Axioskop 2 (Carl Zeiss) with HBO 100 lamps, Axiocam Erc 5s (Carl Zeiss) and ZEN software (Carl Zeiss) with appropriate laser filter for TAMRA (filter 4).

NMR: All NMR experiments were carried out on a Bruker advance III HD 600MHz. NMR spectra were processed in Bruker TopSpin (Delaglio, Grzesiek et al. 1995) and analyzed using Sparky (Lee, Tonelli et al. 2015). Dimer samples of [C^{13},N^{15}]H2A-H2B at 100uM in 5%D_2O/95%H_2O; 25mM NaPi + 100mM NaCl pH6.2 + 0,01% NaN3 + 1mM 2-Mercaptoethano + PIC (complete EDTA-free Protease Inhibitor Cocktail (Roche)) were titrated against GMIP1 using 600MHz Lamour frequency at 308K. HSQC spectra were measured for free [C^{13},N^{15}]H2A-H2B and after the addition of GMIP1 at 308K. Titration consisting of 4 points in the range of 1:4.3 molar ratio ([C^{13},N^{15}]H2A-H2B:GMIP1) was performed.

Circular dichroism: Measurement of secondary structure of NBPeps was performed in Jasco j-815 spectropolarimeter in a 0,1cm quartz cuvette in the range of 190-250nm. Samples were diluted in MiliQ water in the concentration of 0.125mg/mL for GMIP1, LANA, HMGN2pep and H4pep at 0.107mg/mL at 25 ◦C. Data were plotted using BestSel data base (available at: http://bestsel.elte.hu/).

5. RESULTS AND DISCUSSION (NBPeps)

5.1. NBPeps secondary structure characterization

All NBPeps, except for GMIP1, have been well characterized structurally (Luger, Mader et al. 1997, Barbera, Chodaparambil et al. 2006, Kato, van Ingen et al. 2011). Despite GMIP1 being based in the structure of RCC1, the triple alanine bridge connecting the two epitopes created a new structure. For this reason, I performed CD (Circular Dichroism) analysis, as seen in Figure 8.

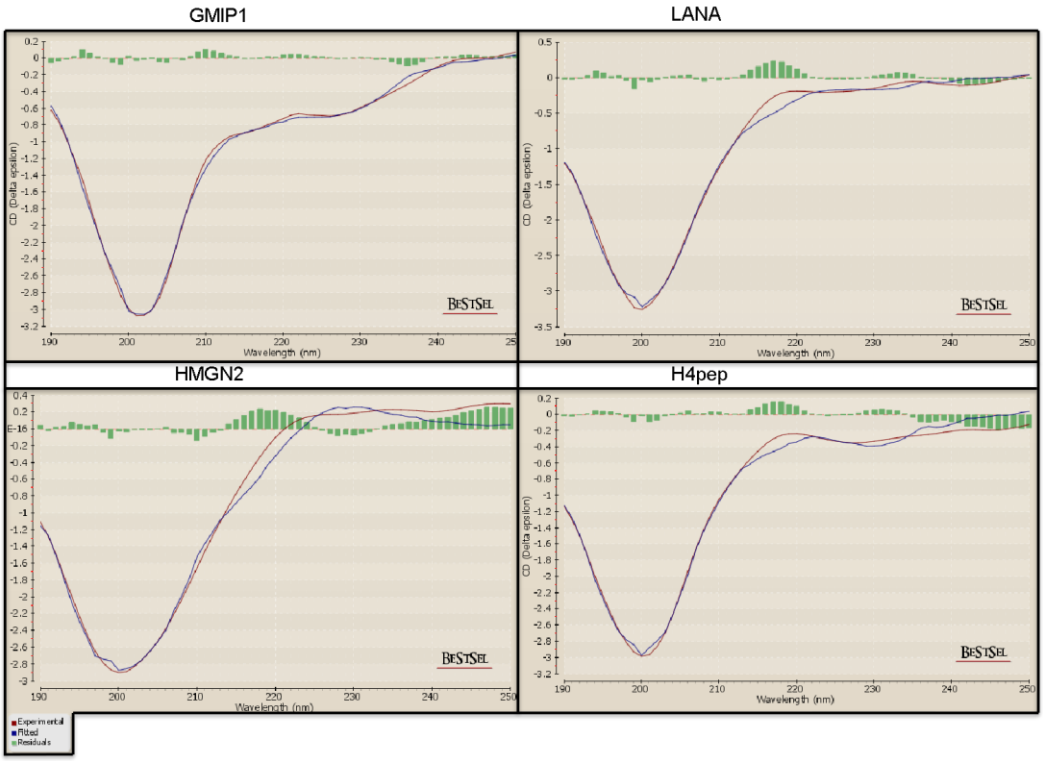

Figure 8: Circular dichroism of NBPeps for determination of secondary structure. All NBPeps show low ellipticity above 210 and negative bands near 195nm, characterizing predominance of random coil structure.

All NBPeps that were developed have no well-defined secondary structure, result which is in agreement with NMR experiments based on the absence of medium or long-range NOEs (Nuclear Overhauser Effect) and random coil 13C chemical shifts for GMIP1 (unpublished data).

5.2. GMIP1 binding to the nucleosome *in vitro*

All NBPeps used, with exception of GMIP1, have their mode of interaction with the nucleosome surface already established at an atomic level, with two distinct modes of binding, (i), LANA and H4pep, that binds exclusively to the acidic patch, and (ii) HMGN2pep and GMIP1 that interacts with nucleosomal DNA and the acidic patch as well. To confirm the epitope of GMIP1, I performed NRM experiments, using isotope labeled dimers of H2A/H2B from *Xenopus Laevis*, as seen in Figure 9a. the binding of GMIP1 should induce changes in the electromagnetic environment of specifics residues in the NRM spectra, causing a shift in the peaks, that was not observed in the dimers even in a molar excess of 4.3 times, also GMIP1 interacted with DNA at a Kd of 50µM or weaker, which is probably in the range of non-specific binding of a charged peptide to DNA, Figure 9b.

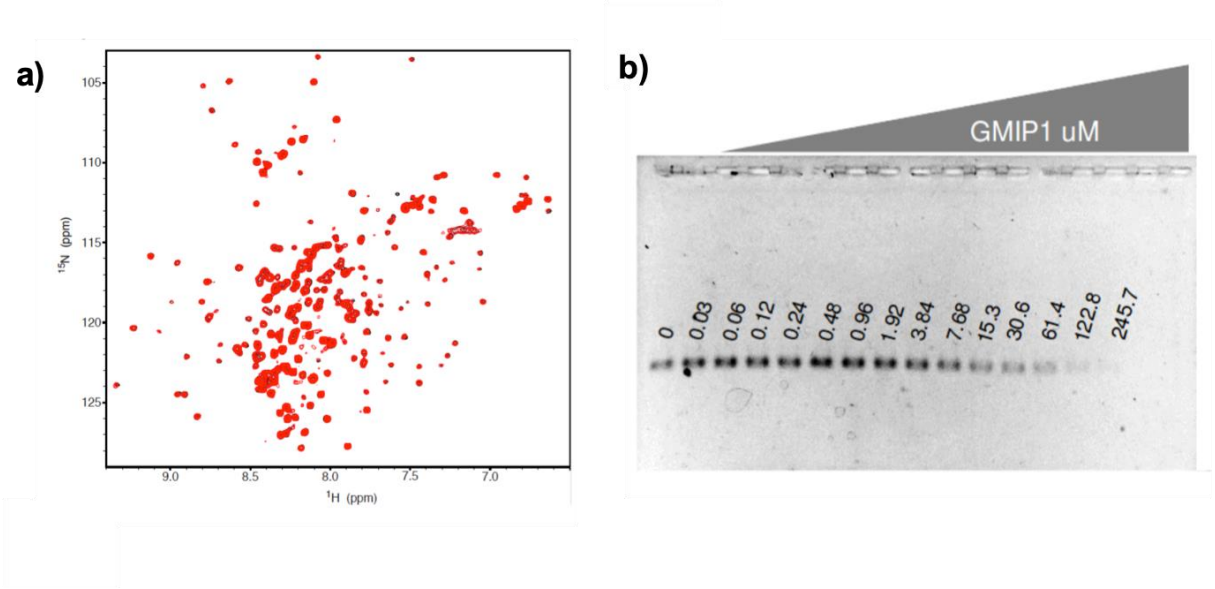

Figure 9: NMR: HSQC spectra of [C13,N15]H2A-H2B dimers (black) and with GMIP1 (red). a) Nucleosomal DNA binding to GMIP1: b) Titration of GMIP1 on the DNA widom 601 (167 bp) analyzed in agarose gel 0,8% in TBE 0.5X. The assay was performed at least 3 times, and the representative gel was presented.

We aimed to create a novel NBPep that could bind to and present specificity for the nucleosome, with high dependence to DNA, that in future projects could be engineered to recognize specific sequences, in a similar manner that was done for transcriptional factors (Desai, Rodionov et al. 2009), giving more specificity to NBPeps with multiple contact sites. However, the biochemical data shows that GMIP1 has low nucleosome binding affinity. It is important to emphasize that all experiments performed were done with the Widom 601 DNA sequence, which is an artificial sequence with high specificity to the octamer.

5.3 NBPeps binding to the nucleosome

Despite most of the NBPeps having its sequences directly derived from NBPs, I wanted to evaluate if it would bind to the nucleosome *in vitro*. In this experiment, I reconstitute nucleosome *in vitro*, with histones from chicken erythrocytes and DNA Widom 601 with 167bp and incubated with the Tagged NBPeps, following analysis in polyacrylamide gel, the gel was then revealed at 510nm so I could visualize the fluorescence from the peptides, the gel was then stained with ethidium bromide and revealed, to show nucleosomal DNA, the bands from the NBPep and the nucleosome were compared to verify if they were at the same height. Peptides were also titrated against a fixed amount of nucleosome, its intensity used to determine the Kd of each NBPep, as seen in Figure 10.

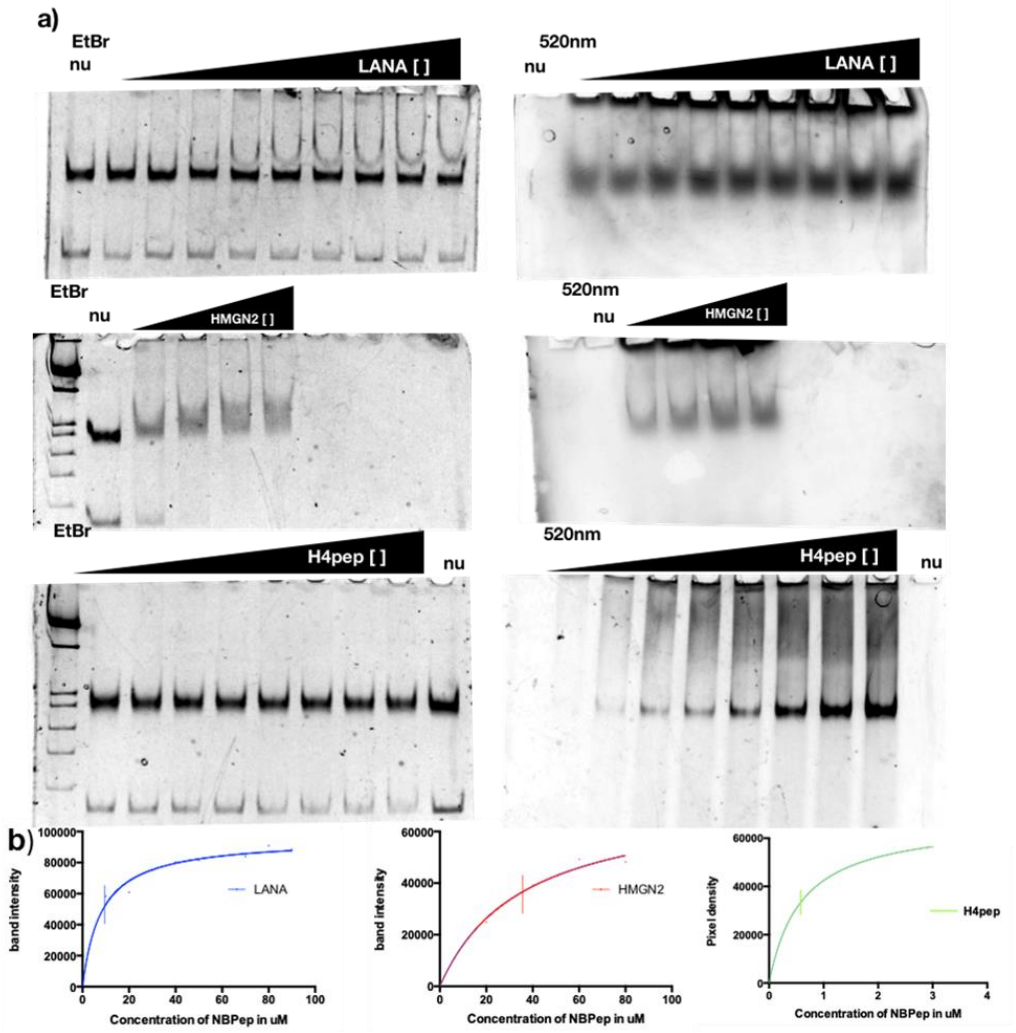

Figure 10: NBPeps interaction assay. a) Nucleosome binding assay with fluorescent NBPeps, nucleosome is incubated with LANA at 0, 10, 20, 30, 40, 50 60, 70 80,90 u M, with HMGN2pep at 0, 20, 40, 60, 80 u M or with H4pep at 0, 200, 400, 600, 800, 1000, 1200, 1400, 1600 nM. It was then analyzed in acrylamide gel, following by detection of the fluorescent NBPep and subsequently detection of DNA. b) Densitometry of NBPeps bands Kd is represented by a vertical line in the densitometry graphs. These assays were performed at least 3 times, and the representative gel was presented.

The NBPeps testes showed a clear band for the peptides at the same height as the nucleosome, furthermore, all presented a dose-response behavior, this data suggests that the NBPeps are binding to the nucleosome in vitro with a Kd of 0.6, 8 and 35uM for H4pep, LANA

and HGMN2pep respectively, GMIP1 induced nucleosome aggregation even at low concentration, as seen in Figure 11, which difficulted to determine the binding affinity constant. HMGN2pep promoted an electrophoretic mobility shift, suggesting that it is binding at more than one site on the nucleosome surface, further investigations will be needed to explore this finding.

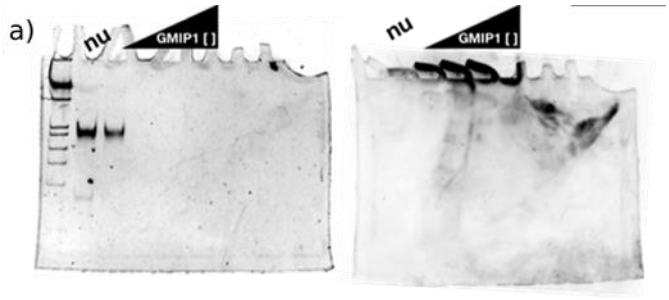

Figure 11: GMIP1 binding assay with nucleosome a) GMIP1 incubated with nucleosome at 0, 50, 100 and 150uM, gel on the left staining with EtBr, to the right gel visualizing the tagged peptide. The assay was performed at least 3 times, and the representative gel was presented.

5.4. NBPeps induce precipitation of the nucleosome in vitro

In order to observe if the fluorescent tag had the impact of the binding of NBPeps to the nucleosome and acquire more information about nucleosome interaction with NBPeps, I performed nucleosome precipitation assay. NBPeps were incubated at different concentrations with freshly reconstituted nucleosomes and samples were centrifuged. The supernatant was transferred to another micro-centrifuge tube, the pellet was resuspended and analyzed in polyacrylamide gel, as seen in Figure 12.

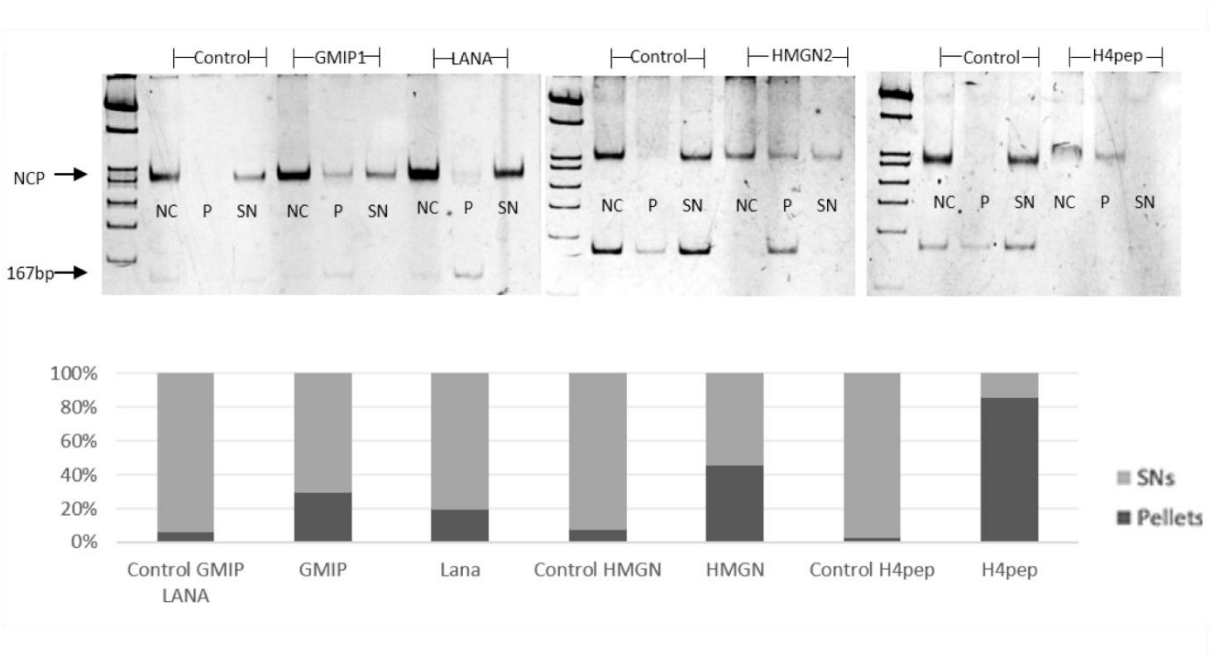

Figure 12: Nucleosome precipitation assay with NBPeps. Nucleosome without NBPeps stays in the supernatant (SN). The addition of 50uM GMIP1, 10uM LANA, 10uM HMGN2pep or 500nM H4pep induce precipitation and Pellet (P) formation. Non-centrifuged (NC) samples were used as control. Densitometry analysis allows a better quantification, with H4pep having the greatest impact.

It was observed that the nucleosome does not precipitate without NBPeps, although all peptides testes induced precipitation at different rates, suggesting binding to the acidic patch and charge neutralization (de Frutos, Raspaud et al. 2001). Notable, H4pep induced precipitation at 500 nM, corroborating to the data from the previous binding assay with fluorescent tagged NBPep.

5.5. NBPeps uptake by cells

In order to bind to the nucleosome, NBPeps must first penetrate the cell to reach the nucleus, one of the issues to use peptides as drugs is that peptides have very low permeability (Shaji and Patole 2008), although there is a class of peptide, known as CPP (Cell Penetrating Peptides) that can cross the cell wall (Prochiantz 2000). CPPs have an overall positive charge and

vary between 5-30 amino acids (Derakhshankhah and Jafari 2018). All NBPeps designed by Dr. Santos' group have these characteristics (see Table 3). Using fluorescent tagged NBPep, I performed flow cytometry analysis in order to quantify and analyze if NBPeps can penetrate cells, and if this is done by passive or active mechanism. See Figure 13.

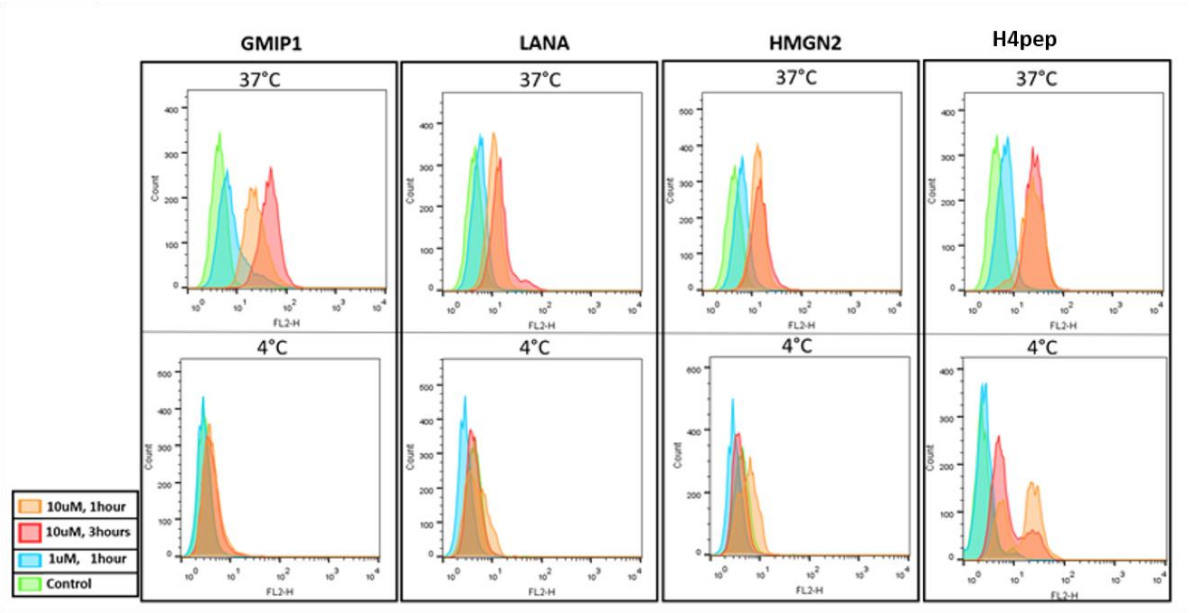

Figure 13: NBPeps cell penetration**.** Flow cytometry profile of Hela cells uptake of fluorescent NBPeps (TAMRA), in histogram view with 1 or 3 hours exposure and at 37 or 4˚C. The assay was performed at least 3 times, and the histogram was presented.

All NBPeps tested penetrated Hela cells, with GMIP1 having the highest uptake. Also by varying the temperature and incubating the cells at 4˚C the active mechanisms of endocytosis in the cell are inhibited (Fernando, Kandel et al. 2010). With the exception of H4pep, all NBPeps are uptake by active mechanisms, with H4pep penetrating both actively and passively.

5.6. NBPeps induce cytotoxicity in a specific manner

In order to verify the cytotoxicity of NBPeps, I performed MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) analysis in Hela and ccd10595k cells. These two types of cells vary significantly, with Hela being an immortalized cell from an aggressive cervical cancer and ccd10595k a primary culture from fibroblasts (Rahbari, Sheahan et al. 2009). As seen in Figure

14, NBPeps have highly distinct cytotoxicity over these two cell lineages.

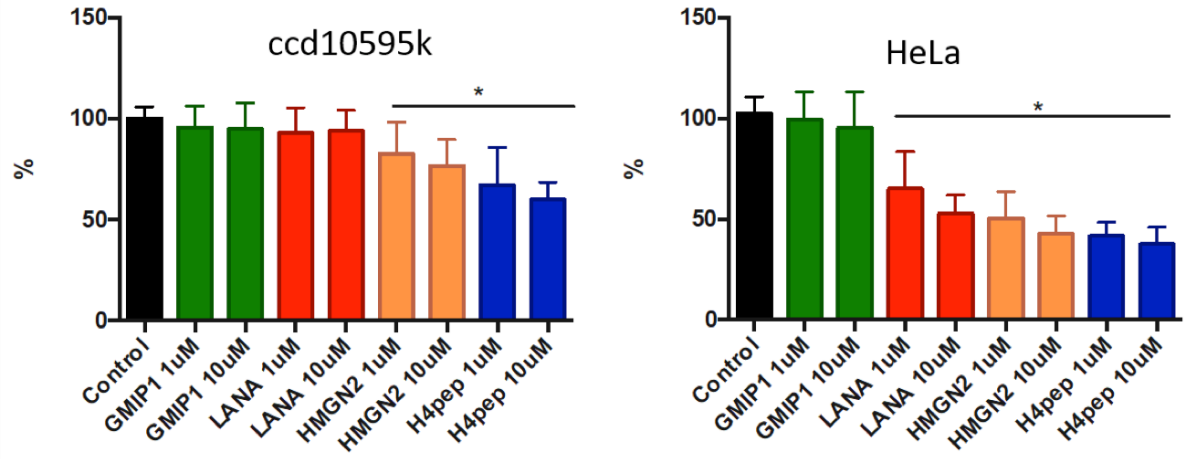

Figure 14: Cytotoxicity evaluation of NBPeps. Relative cell viability compared to control over 24 hours exposure to NBPeps in HeLa and CCD 10595K. HeLa cells showed a greater decreased in cell viability than CCD 10595k for every NBPep tested with the exception of GMIP1. Data is shown as mean ± SD. * represent significant statistical difference (one-way ANOVA test) between the control and treated groups with $= p < 0.05$ and $n = 2$.

For Hela cells, all NBPeps, with the exception of GMIP1 and LANA, showed cytotoxic effects, with a decrease in cell viability greater than 30%. When tested in ccd10595k there were a reduction in cell viability only for H4pep and HGMN2pep, with all other having little to no effect over cell viability. Testing NBPeps in other cell types might help elucidate if cytotoxicity is more prevalent in oncogenic cell lineages or not.

5.7. NBPeps can penetrate cell nucleus *in vivo*

For NBPeps to bind to the nucleosome *in vivo*, it is required to penetrate the cell and reach the nucleus. I evaluated the capability of NBPeps to reach the nucleus *in vivo* using Zebrafish (*Danio rerio)* as a model. The cellular uptake analysis described in section 4.4. could lead to false positives because the NBPeps could be accumulating in the cell membrane or in the cytoplasm. Therefore, I injected adult zebrafish with fluorescent tagged NBPeps, harvested the blood, performed a blood smear, and observed under the fluorescent microscope as seen in Figure 15.

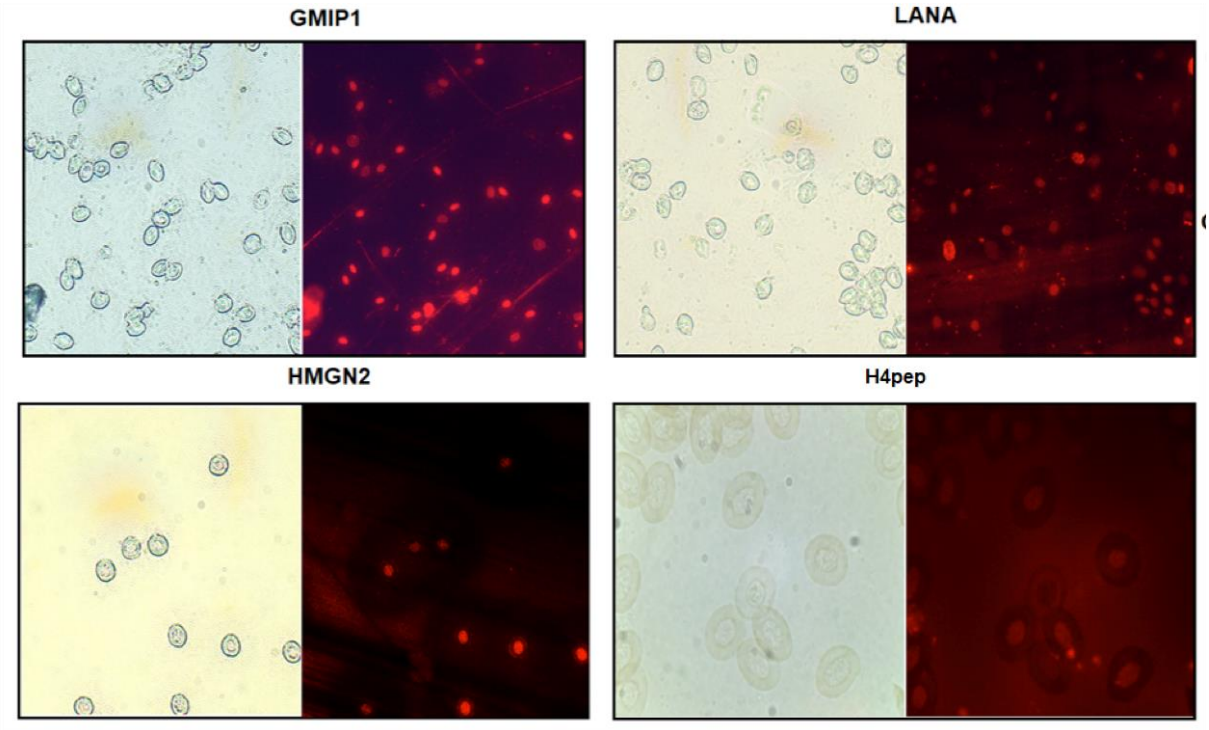

Figure 15: NBPeps distribution *in vivo.* Injection of florescent NBPeps accumulate in the nucleus erythrocytes of adult zebrafish. At the left panel, HBO field, at right panel, visualization using 520 nm laser. The assay was performed at least 3 times, and the representative picture was presented.

It is possible to distinguish very clearly the accumulation of NBPeps in the nucleus, analyzing the formation of a thin halo (cytoplasmic content) around the concentrated red nucleus. This data suggests that NBPeps can reach and accumulate in the nuclear environment *in vivo*.

5.8. NBPeps penetrated different tissues of the zebrafish larvae

NBPeps were designed to bind to every cell with a nucleus, therefore I investigated how NBPeps would be distributed over zebrafish larvae, as seen in Figure 16.

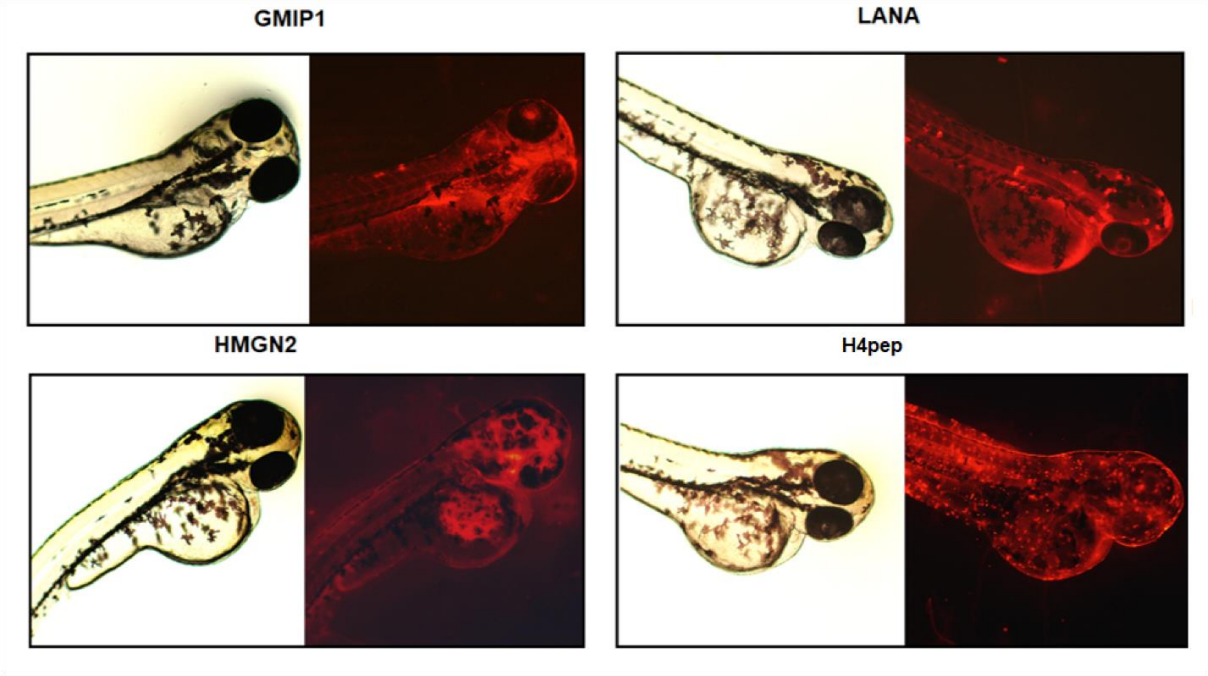

Figure 16: NBPeps distribution in zebrafish larvae with 80hpf. Fluorescent NBPeps incubated for 3h distributes heterogeneously over zebrafish larvae. At the left panel, HBO field, at right panel, visualization using 520 nm laser. The assay was performed at least 3 times, and the representative picture was presented.

The accumulation of NBPeps at the zebrafish larvae penetrated different tissues of the larvae. This result is in agreement with the cell uptake analysis and the blood smear, suggesting that NBPeps can penetrate cells.

5.9. NBPeps induces abnormalities in Zebrafish embryos development

To better understand the effects *in vivo* of NBPeps, I conducted a modified Fish Embryo Toxicity (FET) assay (Morash, Douglas et al. 2011). In my analysis, Zebrafish embryos were incubated with crescent concentrations of NBPeps reaching up to 100uM for 24 hours, at different stages of development, and evaluated for morphological modifications with 1 hour and 24 hours of exposure, see Table 2.

Table 2: Fish Embryo Toxicity assay. Zebrafish embryos at 4, 28 and 52hpf were exposed to NBPeps for 24 hours. The embryos were evaluated for alterations in the development with 1 hour and 24 hours of exposure. H4pep was the only NBPep that induce embryos mortality with 1hour exposure.

24 hours exposure

GMIP 4hpf	n= 3	Alive	Dead	Embryo	Ecloded	Pigmentation	Delay
	0 uM	100%	0%	100%	0%	0%	0%
	0,1uM	96,70%	3,30%	100%	0%	0%	0%
	1uM	100%	0%	100%	0%	0%	0%
	10uM	100%	0%	100%	0%	0%	0%
	100uM	100%	0%	100%	0%	0%	13%

LANA 4hpf	n= 3	Alive	Dead	Embryo	Ecloded	Pigmentation	Delay
	0 uM	100%	0%	100%	0%	0%	0%
	0,1uM	100%	0%	100%	0%	0%	0%
	1uM	100%	0%	100%	0%	0%	0%
	10uM	100%	0%	100%	0%	0%	0%
	100uM	100%	0%	100%	0%	0%	100%

GMIP 28hpf	n=3	Alive	Dead	Embryo	Ecloded	Pigmentation	Delay
	0 uM	100%	0%	100%	0%	0%	0%
	0,1uM	100%	0%	100%	0%	0%	0%
	1uM	100%	0%	100%	0%	0%	0%
	10uM	100%	0%	100%	0%	6%	3%
	100uM	100%	0%	77%	23%	26%	0%

LANA 28hpf	n=3	Alive	Dead	Embryo	Ecloded	Pigmentation	Delay
	0 uM	100%	0%	100%	0%	0%	0%
	0,1uM	100%	0%	100%	0%	3%	0%
	1uM	100%	0%	100%	0%	0%	0%
	10uM	100%	0%	94%	6%	6%	0%
	100uM	90%	10%	100%	0%	90%	66%

GMIP 52hpf	n=3	Alive	Dead	Embryo	Ecloded	Pigmentation	Delay
	0 uM	100%	0%	80%	20%	0%	0%
	0,1uM	100%	0%	84%	16%	3%	0%
	1uM	100%	0%	70%	30%	3%	0%
	10uM	100%	0%	77%	13%	3%	0%
	100uM	97%	3%	14%	86%	10%	0%

LANA 52hpf	n=3	Alive	Dead	Embryo	Ecloded	Pigmentation	Delay
	0 uM	100%	0%	80%	20%	0%	0%
	0,1uM	100%	0%	77%	33%	0%	0%
	1uM	100%	0%	64%	36%	6%	0%
	10uM	100%	0%	60%	40%	6%	0%
	100uM	87%	13%	14%	86%	0%	0%

HMGN2 4hpf n= 3		Alive	Dead	Embryo	Ecloded	Pigmentation	Delay
	0 uM	100%	0%	100%	0%	0%	0%
	0,1uM	100%	0%	100%	0%	0%	0%
	1uM	100%	0%	100%	0%	0%	0%
	10uM	100%	0%	100%	0%	0%	0%
	100uM	75%	25%	100%	0%	0%	0%

H4pep 4hpf	n=3	Alive	Dead	Embryo	Ecloded	Pigmentation	Delay
	0 uM	100%	0%	100%	0%	0%	0%
	0,1uM	100%	0%	100%	0%	0%	0%
	1uM	97%	3%	100%	0%	0%	0%
	10uM	93%	7%	100%	0%	0%	0%
	100uM	0%	100%	100%	0%	0%	0%

HMGN2 28hpf	n=3	Alive	Dead	Embryo	Ecloded	Pigmentation	Delay
	0 uM	100%	0%	100%	0%	0%	0%
	0,1uM	94%	6%	100%	0%	0%	0%
	1uM	100%	0%	100%	0%	0%	0%
	10uM	100%	0%	97%	3%	0%	0%
	100uM	100%	0%	90%	10%	3%	0%

H4pep 28hpf	n=3	Alive	Dead	Embryo	Ecloded	Pigmentation	Delay
	0 uM	100%	0%	100%	0%	0%	0%
	0,1uM	96%	4%	96%	4%	0%	0%
	1uM	100%	0%	96%	4%	0%	0%
	10uM	100%	0%	96%	4%	0%	0%
	100uM	70%	30%	90%	10%	0%	0%

HMGN2 52hpf	n=3	Alive	Dead	Embryo	Ecloded	Pigmentation	Delay
	0 uM	100%	0%	77%	23%	0%	0%
	0,1uM	100%	0%	77%	23%	3%	0%
	1uM	100%	0%	70%	30%	0%	0%
	10uM	100%	0%	40%	60%	16%	0%
	100uM	100%	0%	47%	63%	16%	0%

H4pep 52hpf	n=3	Alive	Dead	Embryo	Ecloded	Pigmentation	Delay
	0 uM	100%	0%	20%	80%	0%	0%
	0,1uM	100%	0%	15%	85%	0%	0%
	1uM	100%	0%	6%	94%	0%	0%
	10uM	94%	6%	6%	94%	0%	0%
	100uM	100%	0%	0%	100%	0%	0%

1 hour exposure

H4pep 4hpf	n=3	Alive	Dead	Embryo	Ecloded	Pigmentation	Delay
	0 uM	100%	0%	100%	0%	0%	0%
	0,1uM	100%	0%	100%	0%	0%	0%
	1uM	100%	0%	100%	0%	0%	0%
	10uM	100%	0%	100%	0%	0%	0%
	100uM	80%	20%	100%	0%	0%	0%

The outcome of embryo exposure to the NBPeps was highly dependent on the stage of development, with the first 28 hours having a higher impact. The hatching rate of zebrafish was largely affected by NBPeps, with GMIP1 having the most pronounced effect, GMIP1 also created defects in the pigmentation in 26% of the embryos and had no impact over mortality. Intriguingly LANA induced delay in the development of 100% on the embryos when they were exposed for 24h with 4hpf and at later stages of development, however, LANA caused no delay in the hatching processes. HMGN2pep affected 16% of the embryos in the development of melanocytes and had a low death rate when compared to H4pep, which was the only one that showed acute toxicity (death with 1houre exposure), also inducing 100% mortality with 24hours of expousere for embryos with 4hpf. This data suggests that NBPeps overall have little toxicity to this animal model, see Figure 17, with increased mortality only at the early stages of development, corroborating the data of MTT in ccd10595 cells.

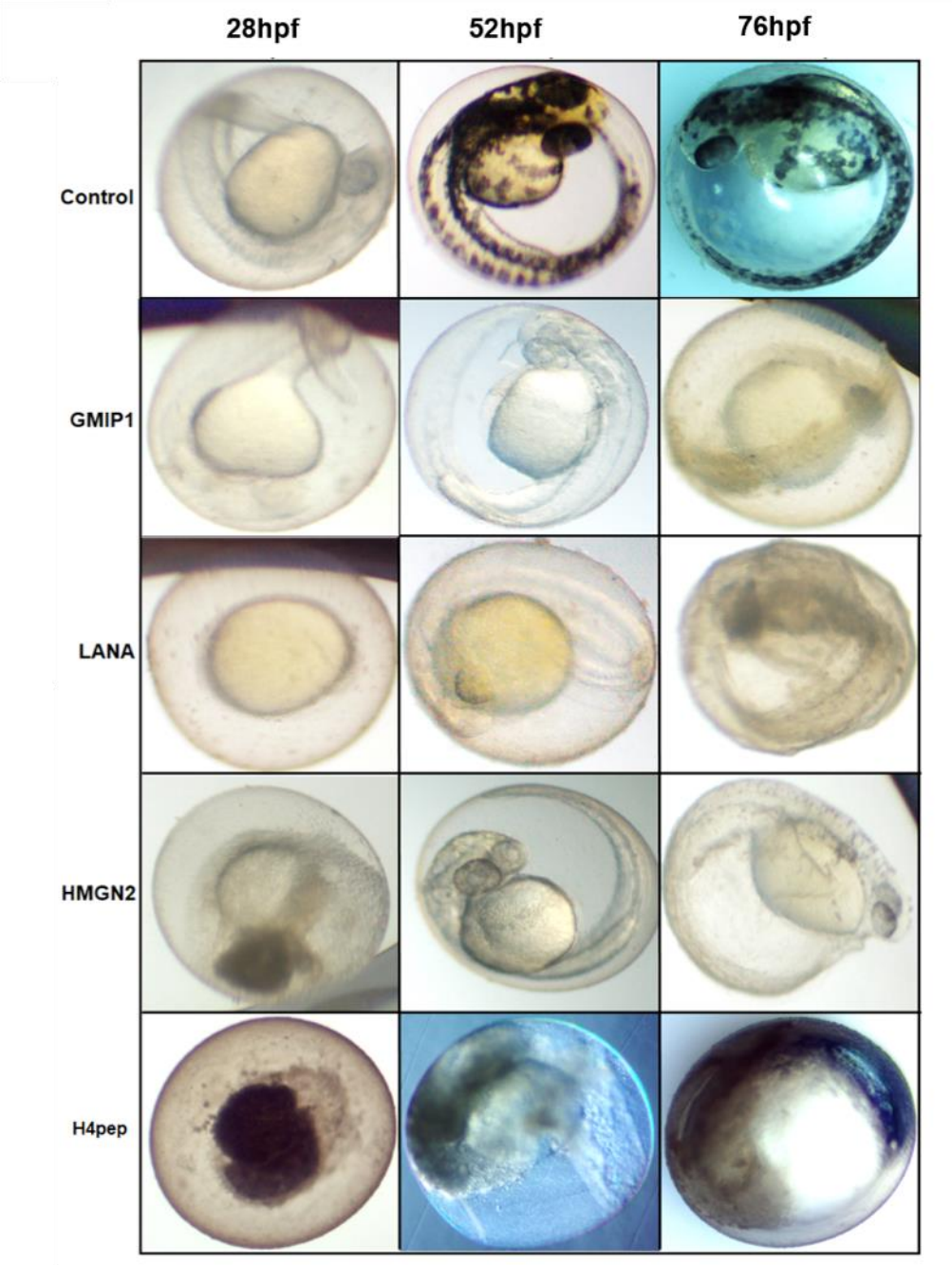

Figure 17: Fish Embryo Toxicity (FET) with NBPeps. Zebrafish embryos at 4, 28 and 52 hours post fertilization (hpf) were incubated with NBPeps or vehicle for 24h. Images are representative of three separate experiments.

The mechanism of how these effects are happening remains unclear, but it is remarkably

interesting to observe that despite the similar binding region of these NBPeps, the effect *in vivo* remains distinct.

These results are not enough to provide a direct correlation between NBPeps binding sites and phenotypic outcomes. Furthermore, only H4pep presented high specificity, with the other three being non-specific nucleosomal interactors, raising the question of whether the NBPeps are not interacting with other chromatin machinery. In fact, Kim and collaborators showed that the tail of histone H4 can be used as a molecular tool to maintain the active state of p53 target genes via interaction with HDAC1 as a novel anticancer therapy (Heo, Kim et al. 2013). Here, I suggest that H4pep could affect tumoral cells, instead of acting only at the modulation of p53 activity, but thought direct nucleosome binding, since it showed higher affinity for the nucleosome

6. CONCLUSION

In conclusion, it was observed that NBPeps can affect the nucleosome structure in multiple ways, despite having a similar target, NBPeps had different effects over cell physiology, which might be due to the non-specificty in targeting the nucleosome surface. However, further experimentation should be performed to be able to correlate the effects of NBPeps binding sites with the physiological outcome. Despite the pioneering work done here, not all aspects that cover the modulation of chromatin via NBPeps were elucidated.

Nevertheless, considering that there are several pharmacological agents, such as DNA intercalators, with great relevance to the clinical practice, the fact that NBPeps are not specific would not preclude their potential as therapeutic agents. Taken all together, I believe that NBPeps open novel opportunities to design hybrid molecules with higher specificity to regulate a plethora of cellular disorders.

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CHAPTER II

8. INTRODUCTION PART II - LIPIDS

Lipids, along with proteins, nucleic acids, and carbohydrates are macromolecules with a myriad of functions in biological systems. Due to its great diversity on structure and function, lipids received the broad definition of "biological substances that are generally hydrophobic in nature and in many cases soluble in organic solvents (Cammack, Atwood et al. 2008)". This definition covers a large range of structurally distinct molecules, such as fatty acids, phospholipids, steroids, sphingolipids, terpenes, and others, with very distinct functions (Berry 2004, Fahy, Subramaniam et al. 2009, Subramaniam, Fahy et al. 2011). For example, phospholipids are molecules with two hydrophobic tails and a hydrophilic head, this enables phospholipids to form bilayered membranes, which cause them to be the most prevalent component in cell membranes in eukaryotes and procaryotes, also phospholipids can be used as a tool for drug delivery (Li, Wang et al. 2015). On the other hand, steroids are recognizable by their steroidal rings, which are composed of three cyclo-hexanes and a cyclo-pentane, they have important functions in cell signaling, with sex hormones being one example, also, steroids are an important component of cellular membranes, modulating membrane permeability (IUPAC-IUBMB 1998). For the purpose of this work I will focus on lipids present in the nucleus of Eukaryotes with a focus on the chromatin.

8.1. LIPIDS IN THE NUCLEUS

It was thought that the nuclear environment was composed mainly of DNA, RNA, histones, and other proteins (Albi and Viola Magni 2004). However, in 1939 the first evidence of lipids in the nucleus was shown by Stoneburg. Using rabbits and rats, he was able to determine the concentration of several lipids chemo-types in the nucleus (Stoneburg 1939). Later the evidence for phospholipids in the nuclei of liver cells was demonstrated (Chayen and Gahan 1958). In 1999, Donnelley and collaborators established there is a relationship between nuclear lipids content and cell cycle regulation in plants, giving new insights about the role of lipids in the nucleus (Donnelly, Bonetta et al. 1999). These processes were later shown in detail in animal cells using LC-MS techniques (Atilla-Gokcumen, Muro et al. 2014).

It is also known that lipids in the nucleus can associate with proteins and form distinct structures such as lipids microdomains. These domains harbor several enzymes related to lipids biosynthesis, metabolism and can be a platform for the transcriptional process (Cascianelli, Villani et al. 2008). Further functions of lipids in the nucleus have been observed, such as ligands of nuclear receptors, thus regulating gene expression (Warner, Huang et al. 2017).

Another form of lipid organization in the nucleus is the formation of nuclear Lipids Droplets (nLD). Formed mostly by neutral lipids, they appear as small dots under the light microscope that can be stained with Sudan red. Initially, the formation of nLD was thought to be due to the entrapment of cLD (Cytoplasmatic Lipids Droplets), with this phenomenon occurring randomly or as a reservoir for other lipids in the nucleus (Layerenza, Gonzalez et al. 2013). However it was observed that nLD were more frequent in hepatocytes, which do not present high levels of cLD (Sołtysik, Ohsaki et al. 2019), to further raise doubt in this issue, adrenocortical cells, that are rich in cLD do not present abundance of nLD (Ohsaki, Kawai et al. 2016). However, recent studies in rats and yeast, showed that nLD have a regulatory function in phosphatidylcholine synthesis, nevertheless the mechanism by which the formation of nLD occurs remains elusive (Romanauska and Kohler 2018, Sołtysik, Ohsaki et al. 2019).

Lipids also have important roles in the regulation of proteins that compose the nuclear envelope. Farnesylation and geranylgeranylation are covalent reactions that add the lipids into cysteines, these reactions are responsible for anchoring many small GTPases in at the plasma membrane, for example (Young, Fong et al. 2005). Lamins are the most abundant type of proteins in the nucleus (Schwanhausser, Busse et al. 2011), it has been identified that disruption of the normal processing of the lamin A farnesylation is involved in several diseases, such as Atypical Werner's Syndrome and Hutchison-Gilford Progeria Syndrome (HGPS) (De Sandre-Giovannoli, Bernard et al. 2003, Eriksson, Brown et al. 2003, Burke and Stewart 2013, Zhdanov, Schirmer et al. 2016), highlighting the importance of proper lipid processing in the nuclear environment, Figure 18 shows the main compositions of some nuclear compartments and the targets of lipids as PTMs.

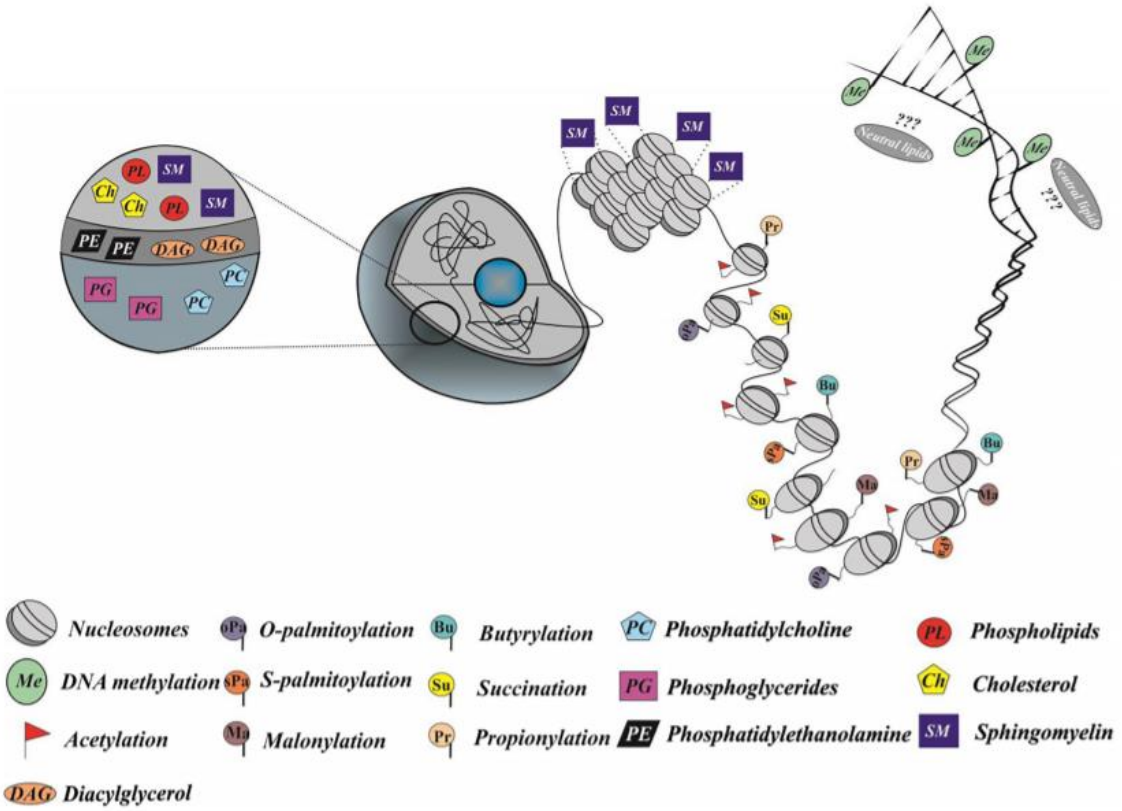

Figure 18: Distribution of lipids in the nucleus. A nucleus highlighting the major lipids found in the nucleoplasm, nuclear membrane (left), and chromatin fibers (right). Lipid moieties found to be present in PTMs in core histones. Lipid types and lipid chromatin modifications are listed above. (Adapted from :(Zhdanov, Schirmer et al. 2016))

8.2. LIPIDS, DNA, AND CHROMATIN

Phospholipids, as said in previous sections, are a major component of cellular membranes, however, their function in the cell is not limited to structural functions, also they are important components in chromosomes, chromatin, and nuclear matrix (Martelli, Capitani et al. 1999, Struchkov and Strazhevskaya 2000). The first evidence of lipids interacting with DNA was shown by Kuzin and colleagues, using rat thymus and livers, they isolated DNA bound to lipids (Belyaev, Strazhevskaya et al. 1974). Further studies characterized the lipid bound DNA in two categories, (i) loosely bound lipids and (ii) tightly bound lipids. The fraction (i) received this name because

lipids were easily washed from DNA with 35% ethanol, and composed ~60% of all lipids bound to DNA. The fraction (ii) was treated with DNase 1 for one hour and extracted with methanol chloroform2:1 solution treatment. The two pools of lipids extracted from DNA had a different quantitative composition of neutral lipids and specifics fatty acids, with this quantitation being highly dependent on cell type and cell cycle (Struchkov, Strazhevskaya et al. 2002).

The first evidence of lipids bound to the chromatin was shown by Erickson and collaborators (Erickson, Davison et al. 1975). Using labeled cholesterol, they identified bound to the chromatin. Nine years later, it was shown that this binding was trough one or more proteins and not trough the DNA (Regenass-Klotz and Heiniger 1984). In 2017, Dr. Santos' group published a paper showing that the cholesterol assists in the 10 and 30 nm chromatin formation and induces folding of long chromatin fibers as a result of direct interaction of the cholesterol to six nucleosomal binding sites (Silva, Fernandes et al. 2017). There are other small lipid molecules are present in the nucleus (Zaina et al., 2005), raising questions about other lipids and their interaction with the chromatin.

Lipids have been identified regulating chromatin machinery, such as the case of EPA (eicosapentaenoic acid), that showed inhibitory activities over DNMT (DNA methyltransferase) and HDAC1. In hepatocarcinoma cells, EPA binds to PPAR-γ (Peroxisome proliferator-activated receptor gamma) causing the downregulation of HDAC1 and DNMT, thus showing promissory effects in tumor suppression (Ceccarelli, Ronchetti et al. 2020).

Since de discovery that Hp1α induces chromatin compaction by phase separation (Larson, Elnatan et al. 2017, Strom, Emelyanov et al. 2017), and further experiments showing that histone tails and its acetylation status are also regulating phase separation (Gibson, Doolittle et al. 2019), the idea of droplets out of solution, like oil de-mixing in water, might have important regulatory functions over the chromatin. Despite increasing knowledge about lipids in the nucleus and chromatin, the role of this class of ubiquitous molecules remains unclear at the chromatin level. In this work we review data about lipids in the nucleus and suggest that other lipids, not only cholesterol, may have regulatory functions at the chromatin level.

9. AIMS PART II - LIPIDS

9.1. PRIMARY AIMS

Review the key impacts of lipids on the nuclear environment, emphasizing its role on chromatin architecture.

9.2. SECONDARY AIMS

- Identify the lipids in the nuclear environment;
- Discuss the potential roles of nuclear lipids on chromatin architecture.

10. METHODS

Lipidic profile of nuclear compartments in liver, thymus, and embryonic cells: For this work I reviewed the literature for Lipids AND Chromatin; Lipids AND Nucleus; Lipids AND Nuclear Environment. I then selected relevant papers for the subject and compiled the usable data. Papers using similar methodologies and cell types were used. Data of lipidic content in cells is expressed in relation to protein:lipid or protein:tissue. Thymus and liver were selected due to the abundance of good data available. Normalization was done using the most prevalent lipid-chemotype as 100% and others in relationship to it using Graphad Prism6 software.

11. RESULTS AND DISCUSSION (Lipids)

In our review entitled "Fat nucleosome: Role of lipids on chromatin" and published at Progress Lipid Research in 2018, see appendix B (Fernandes, Teles et al. 2018), we looked at lipids as a new modulator for the chromatin, evaluating the lipid content at different nuclear environment and cellular types, lipids involved in gene expression, lipids with potential to form complexes with the nucleosome and clinical outcomes of lipids and chromatin interaction.

11.1. Lipids in the nuclear environment: lipids and chromatin

The nuclear environment varies significantly among cell types, especially during cell cycle, making the analysis of lipids in the nuclear environment a difficult task (Kolomiytseva, Kulagina et al. 2002, Zhdanov, Schirmer et al. 2016). Nonetheless we were able to compile data from several studies determining the concentration of lipids in the nuclear environment, as seen in Figure 19. We also compared different nuclear compartments of iMEF (immortalized murine embryonic fibroblasts), which were thoroughly analyzed by ESI-MS and provided outstanding data (Tribble, Ivanova et al. 2016).

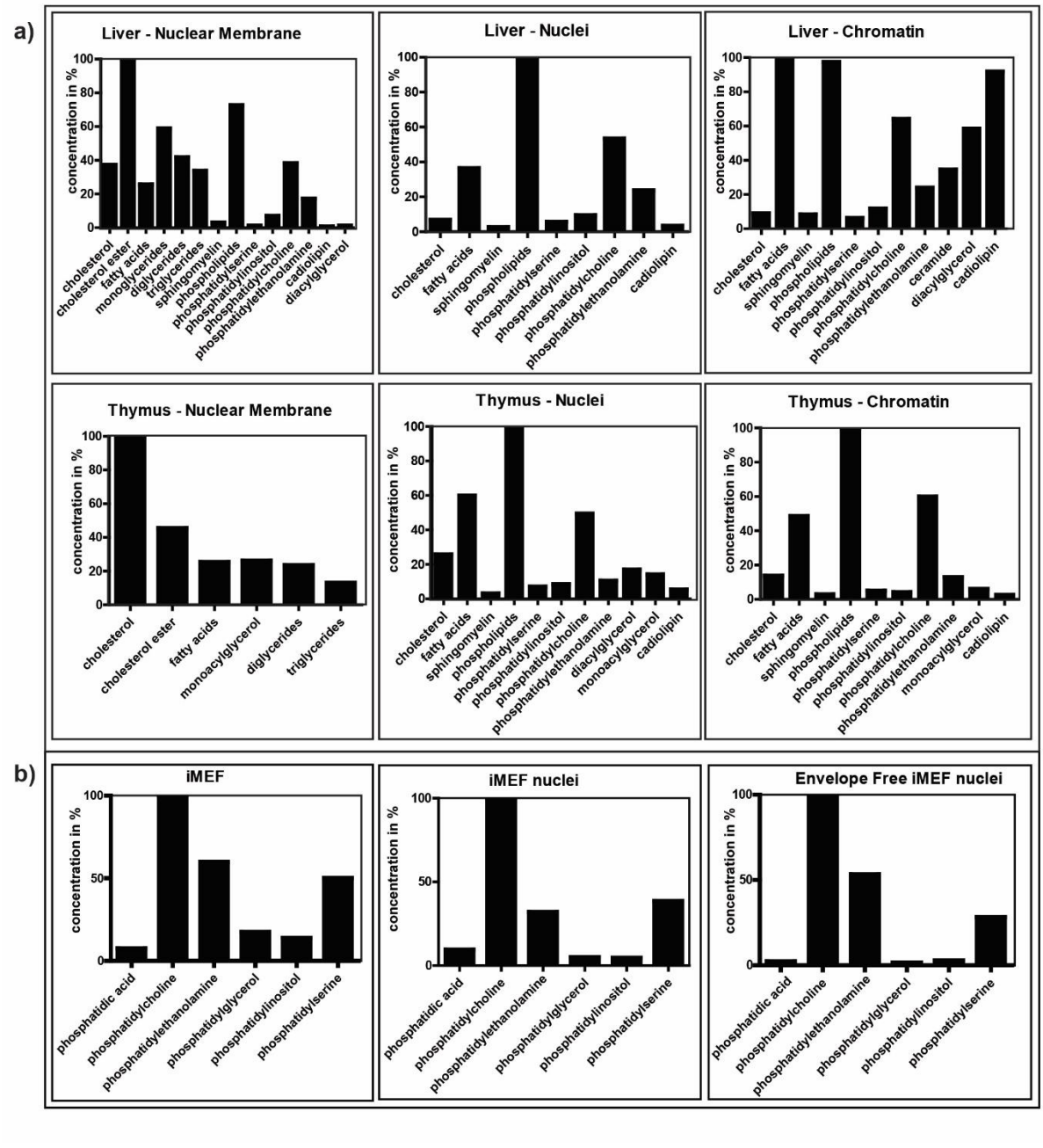

Figure 19: Lipidic profile of nuclear compartments in liver, thymus, and embryonic cells. a) The concentration of lipid is expressed in relation to protein:tissue, normalized in percentage related to the highest lipid concentration, from liver and thymus cells. b) Nucleus of iMEF cells, includes data from whole nuclear environment, including chromatin and nuclear membrane.

With the available literature, it was possible to compare three distinct nuclear environments

of two distinct tissues (Fig 19 a). Although phospholipids are a major component of all nuclear environments, there are many differences in the lipidic composition, such as the difference in cardiolipin in the thymus and liver chromatin. This suggests that lipid distribution in the nucleus is cell dependent and specific roles of these lipids at the chromatin still to be elucidated. Also, in Figure 19 b, it is possible to observe that the content of phospholipids in iMEF (immortalized murine embryonic fibroblasts) cells do not vary considerably among the whole cell, the nucleus and the nuclear envelope. Following the trend in liver and thymus cells, phosphatidylcholine is the most abundant phospholipid overall.

12. CONCLUDING REMARKS

It is known a great deal about how chromatin is modulated by NBPs, however, the understanding about other types of molecules, for example the lipids, is not as clear. New insights about the role of cholesterol interaction with nucleosome suggest a new function for lipids as modulators of chromatin (Silva, Fernandes et al. 2017). *In silico* studies conducted by Fernandes, V. shows that the nucleosome surface has several binding sites for lipids, with low affinity (unpublished data). This might suggest that the nucleosome surface may work as a reservoir of lipids overall and that it might influence the final chromatin status.

Summarizing some of the new insights from our review, we created a cartoon illustrating new functions that lipids may have over the nucleosome, as seen in Figure 20.

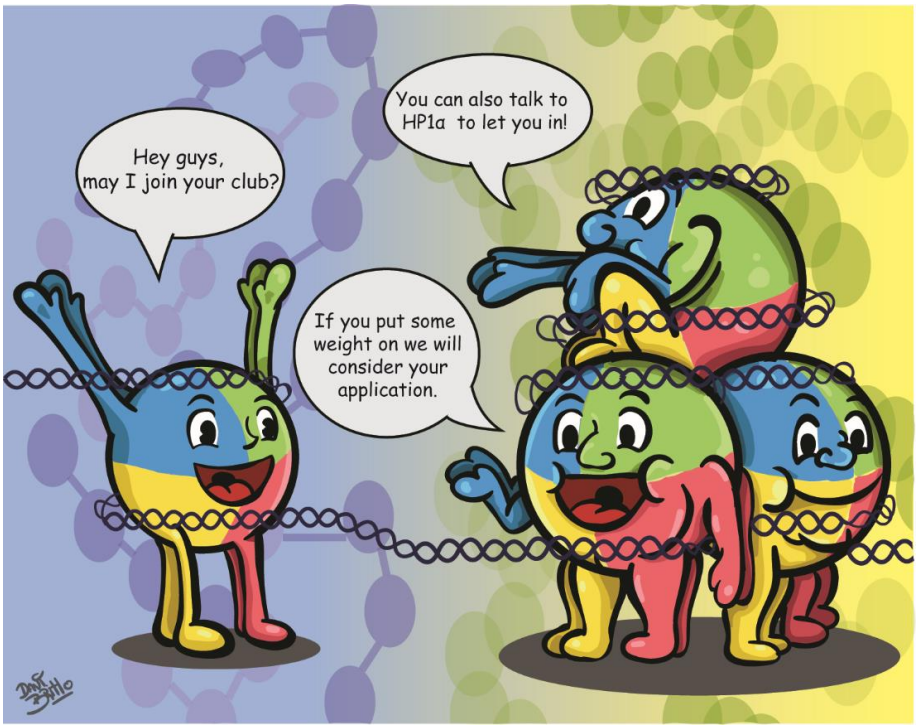

Figure 20: Hypothetical role of lipids on chromatin. Heterochromatin formation is the condensed state of chromatin, which can be induced by HP1α (heterochromatin protein 1 alpha) and lipids, potently through phase-separation process. The nuclear environment is represented in different colors, where internucleosomal interaction (three lipid-bound nucleosomes) is favored in a phase-separated compartment.

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CHAPTER III

14. INTRODUCTION PART III - PHASE SEPARATION

The mechanism of phase separation seems to be present in a diverse range of the gene regulation machinery, such as enhancer activity and eRNA production (Hnisz, Shrinivas et al. 2017), the interaction of transcriptional factors OCT4 (octamer-binding transcription factor 4) and GCN4 (General control protein GCN4) with Mediator and p300 (Boija, Klein et al. 2018), how Polycomb Repressive Complex 1 (PRC1) and HP1α regulates chromatin compaction (Larson, Elnatan et al. 2017, Strom, Emelyanov et al. 2017, Plys, Davis et al. 2019), this suggests that phase separation is a common feature in chromatin regulation.

14.1 Phase separation

The eukaryotic cell is organized in compartments, such is the nuclear membrane, that works as a physical barrier helping organize the nuclear content, selecting molecules that can enter or exit the nuclear environment, thus regulating cellular function. There are several other membrane-bound organelles, such as lysosomes, mitochondria, endoplasmic reticulum and others. Nevertheless, some organelles are not dependent on a membrane delimiting its volume, these are known as membranelles organelles, nucleolus, nuclear speckles, SGs (Stress Granules), processing bodies and the centriole are some examples (Uversky 2017). Despite the discovery of these membranelles organelles several years ago, how they form, their physical proprieties and their contribution to biological functions remained elusive (Boeynaems, Alberti et al. 2018).

The first description that membraneless organelles are formed by phase separation was revealed in 2009 by Brangwynne and collaborators, they showed that of P granules have liquid-like properties, describing that P granules are dynamic, with fusion and fission events, in a similar fashion to oil droplets in water (Brangwynne, Eckmann et al. 2009). Despite that being the first evidence of phase separation in cells, the phenomena have been already observed *in vitro.* In 1991, researchers had observed that high concentrations of Haemoglobin can create a binary-liquid phase separation, however, the biological relevance of these results remained unclear (Broide, Berland et al. 1991).

Recent studies have shown that multivalency of adhesive domains and/or linear motifs is a common feature among proteins that can phase separate (Boke, Ruer et al. 2016, Jain, Wheeler et

al. 2016, Boeynaems, Alberti et al. 2018). The multivalency can explain phase separation in three distinct mechanisms: (i) folded proteins with well-defined interaction surfaces; (ii) folded domains connected by flexible linkers; and (iii) IDRs (intrinsically disordered regions), that are sections of the proteins that do not adopt secondary or tertiary structure, are dynamic, heterogeneous and have repetitive amino acids in their sequence (Mitrea and Kriwacki 2016), IDRs can create short interaction motifs, all these criteria can happen in alone or in combination to form a phase separated environment (Boeynaems, Alberti et al. 2018).

The formation of phase separation can happen in distinct phases, it can form a LLPS (Liquid-Liquid Phase separation), a hydro gel like phase separation or the formation of solids states, the physical state of the environment is extremely relevant for the function of this organelles, see Figure 21.

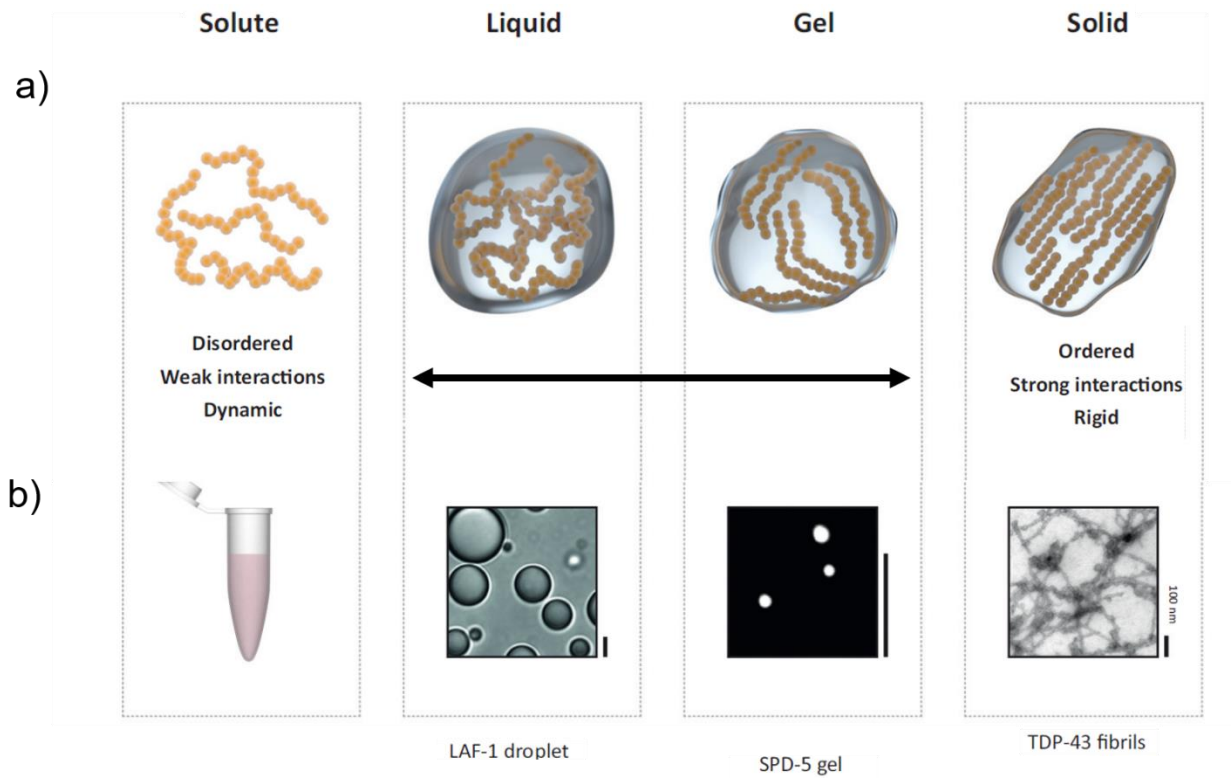

Figure 21: Phase separation and its physical states. (a) Three kinds of phase separated environment

and their respective molecular arrangement. (b) Different proteins can create 3 distinct types of phase separation *in vitro*. Adapted from (Patel, Lee et al. 2015, Boeynaems, Alberti et al. 2018)

When the formation of the phase separated environment occurs, it creates an enriched region with specifics molecules that is shielded from the other molecules, this can be especially useful for virus, in fact, that is the strategy that adopted by the rabies virus, which creates a Negri body, that is, a LLPS inside the cell cytoplasm, that works as a viral factory and can shield itself from the undesired cellular machinery (Nikolic, Le Bars et al. 2017). In fact, several diseases have been linked to the phase separation phenomenon such as ALS (Amyotrophic Lateral Sclerosis) and FTD (Frontotemporal Dementia) (Alberti and Dormann 2019). Mutation in RNA binding proteins cause them to accumulate in membraneless organelles known as SGs, that further can evolve to pathological RNA-binding protein aggregates, these aggregates are found post mortem in the brains of patients with ALS and FTD (Liu-Yesucevitz, Bilgutay et al. 2010, Dewey, Cenik et al. 2011, Alami, Smith et al. 2014, Gopal, Nirschl et al. 2017, Mackenzie, Nicholson et al. 2017).

14.2 Histone like motifs

Histones like motifs are lysine rich sequences that are present in the tails of histones H2A and H4 (Yarychkivska, Shahabuddin et al. 2018), this motif is also found in other chromatin acting proteins, such as p53, HsfB1, Sall1-4, DNMT1 and are related to acetylation processes (Gu and Roeder 1997, Bharti, Von Koskull-Doring et al. 2004, Koyama and Kurumizaka 2018, Yarychkivska, Shahabuddin et al. 2018). The lysin rich sequences are a target for several epigenetic writers such as HAT class of enzymes (Legube and Trouche 2003), and are known to regulate these proteins (Bharti, Von Koskull-Doring et al. 2004, Du, Song et al. 2010). Furthermore, IDRs, are found in histone like motifs (Mitrea and Kriwacki 2016).

Data acquired during the experiments with NBPeps and nucleosome precipitation, see Figure 12, was intriguing, the band resulting from the complex, nucleosome and H4pep appears smeared, which is unusual for the interaction with a small peptide, therefore giving that the nucleosome is largely regulated by phase separation, I investigate if the NBPep, H4pep have the propriety of phase separation.

15.AIMS PART III – PHASE SEPARATION

15.1. PRIMARY AIMS

The goal of this study is to investigate *in vitro* the role o a nucleosome binding peptide derived from H4 tail (H4pep) in the phase separation phenomenon.

15.2. SECONDARY AIMS

- Investigate if DNA is required for the formation of phase separation
- Investigate the type of phase separation involved in H4pep:DNA interaction
- Investigate the time dependency in the phase separation phenomenon H4pep:DNA complex

16.METHODS

DNA widom 601: DNA containing the Widom 601 sequence with 167bp was obtained using plasmid donated from Dr. Rhodes. The plasmids were transformed in competent cells using heat shock technique, as described in (Froger and Hall 2007). Cells were grown in media containing ampicillin and further the plasmid was extracted using Maxprep (Laboratory of Molecular Pharmacology protocols). Further 1mg of plasmid was digesting 5U/ug of DNA using the restriction enzyme AVAI (New England Biolabs) overnight at 37 degrees Celsius, the reaction was stopped by adding EDTA to the final concentration of 10mM. The 167bp DNA was isolated from the rest of plasmid by using 30% PEG (polyethylene glycol) 4000, 1,5M NaCl, incubating with the DNA, the DNA was centrifuged (Sigma centrifuge-2K15) at 15493 x g for 10 minutes at 25 °C, the supernatant was transferred to another microcentrifuge tube and the pellet was resuspended in the MiliQ water and both were analyzed in 1% agarose gel, the processes was repeated until no DNA fragment was found in the supernatant. The purified DNA was then cleaned using phenol/chloroform method and then quantified using Nanovue (GE) to the final concentration adjusted for 1ug/uL.

Droplet assay: Specified amounts of selected NBPeps were incubated with DNA Widom 601 with 167bp for the specified amount of time in LLPS buffer (25mM HEPES pH 7.5, 150mM NaCl, and 1mM DTT). Droplets were mounted in a visualization chamber with silanized glass (see Results and Discussion.

Droplet aging assay: Specified amount of H4pep was incubated with DNA Widom 601 with 167bp for the specified amount of time in LLPS buffer (25mM HEPES pH 7.5, 150mM NaCl, and 1mM DTT). Droplets were mounted in a visualization chamber with silanized glass (see Results and Discussion) and observed under a DIC (Differential interference contrast) microscope.

Salt resistance assay: Droplets were prepared by mixing to a final concentration of H4pep of 30uM with 0.1ug/uL of DNA Widom 601 with 167bp in LLPS buffer with specified amounts of NaCl. Samples were incubated for 10 minutes at room temperature and visualized under a DIC microscope.

17. RESULTS AND DISCUSSION

As said previously, the investigation of phase separation in the context of H4pep started with the results obtained in the nucleosome precipitation assay, described in chapter one, I then proceeded to investigate if H4pep could induce phase separation and what were the requirements for it. Using the methodology described in (Alberti, Saha et al. 2018) and (Wang, Zhang et al. 2018) which contains methods to analyze phase separation in the context of macromolecules. Therefore I performed droplet assays under DIC (Differential interference contrast) microscope, in order to avoid evaporation and to diminish the issues with focal points, a visualization chamber assembled, as described in (Alberti, Saha et al. 2018), also, all the glassware were silanized using the methods described in (Szkop, Kliszcz et al. 2018).

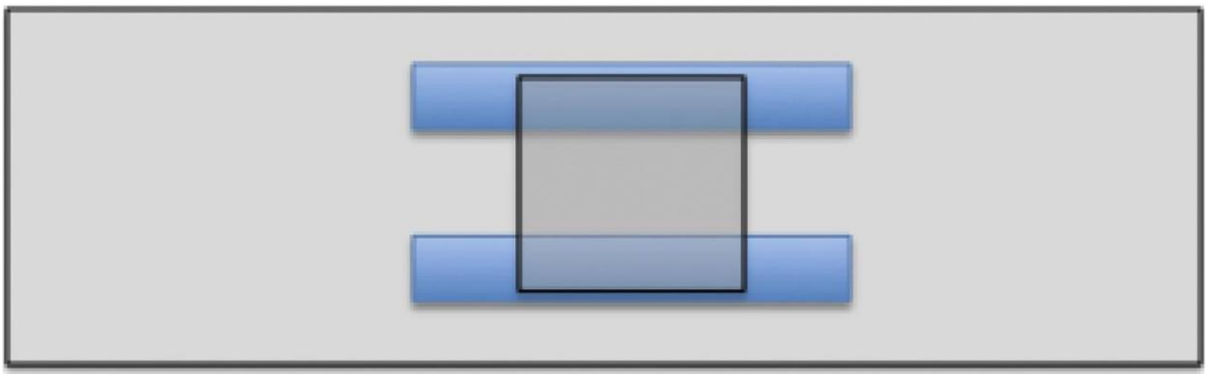

Figure 22: Visualization chamber for droplet assay. Droplets of 10uL were pipetted in the silanized glass surface and spacers were placed using 3M tape followed by another silanized glass, trapping the droplet and preventing evaporation. Adapted from (Alberti, Saha et al. 2018).

17.1 H4pep is dependent on DNA to the formation of phase separation

I performed the droplet assays with H4pep and LANA, which have shown no band smearing in the assay of nucleosome precipitation, as a control. First, I screened to see if the H4pep would phase separate by itself, however, at concentrations of 90uM no effect was observed, LANA showed similar effects. Several proteins that phase separate requires biding partners to create the microenvironment, therefore I decided to investigate if DNA could have any effect over the phenomenon. DNA it is known to bind to the histone H4 tail (Sinha and Shogren-Knaak 2010), as

said previously, the H4pep is directly derived from the histone H4 tail, also the data showed in chapter one, strongly suggests that DNA can bind to the H4pep avidly. Hence when DNA was added at 0.1ug/uL, at 30uM of H4pep, it was possible to observe the formation of spherical droplets, suggesting the formation of phase separation, at higher concentrations of H4pep, it was observed the formation of precipitates, see Figure 23.

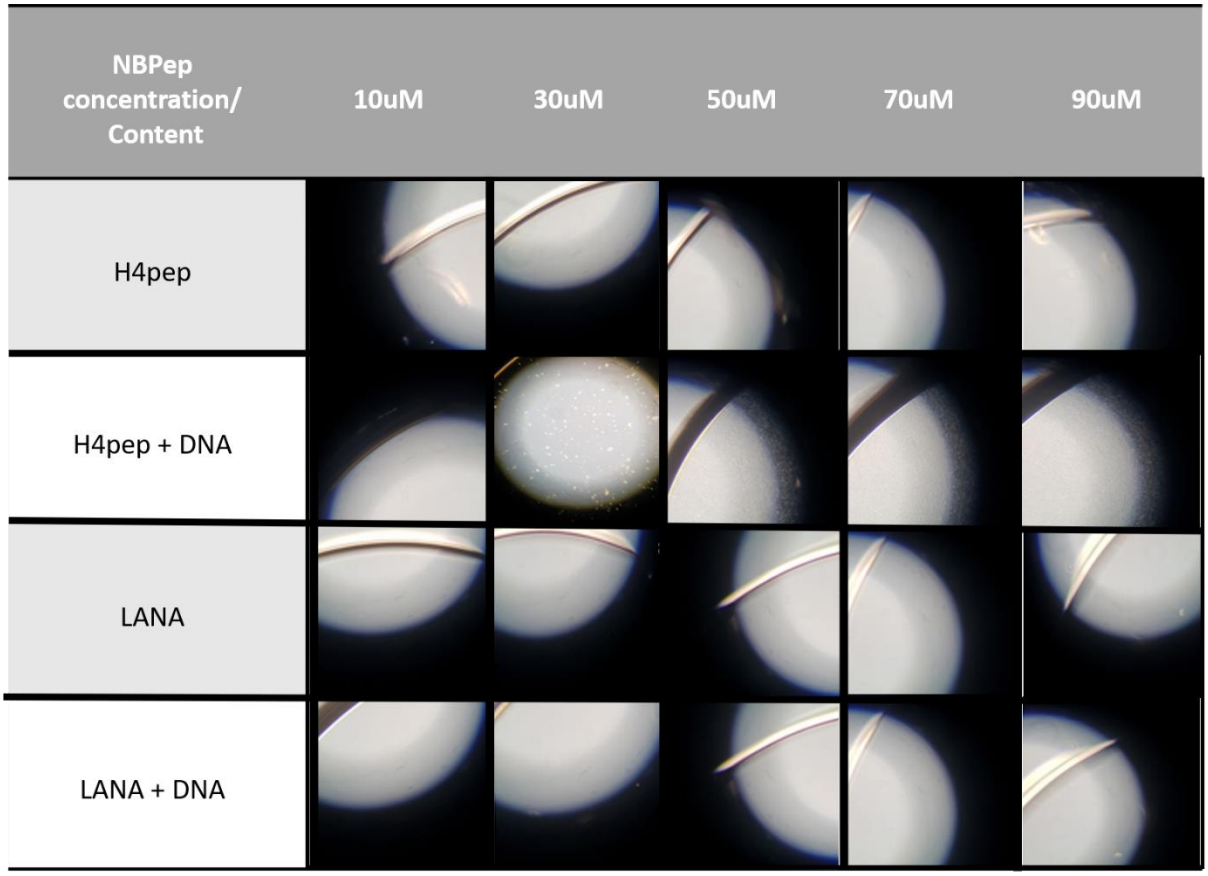

Figure 23: Droplet assays. H4pep was screened to determine the optimal concentration for the formation of phase separation and the required binding partners, LANA was used as a control. Images are representative of three separate experiments.

17.2 Kinetics of phase separation of H4pep

The kinetics of phase separation have important biological relevance, for example, age-related neurodegenerative diseases are linked to aberrant phase transitions in neurons and other diseases (Murakami, Qamar et al. 2015, Alberti and Hyman 2016). Therefore, I decided to

investigate the impact that time has on the formation of the phase separation driven by H4pep and DNA, see Figure 24.

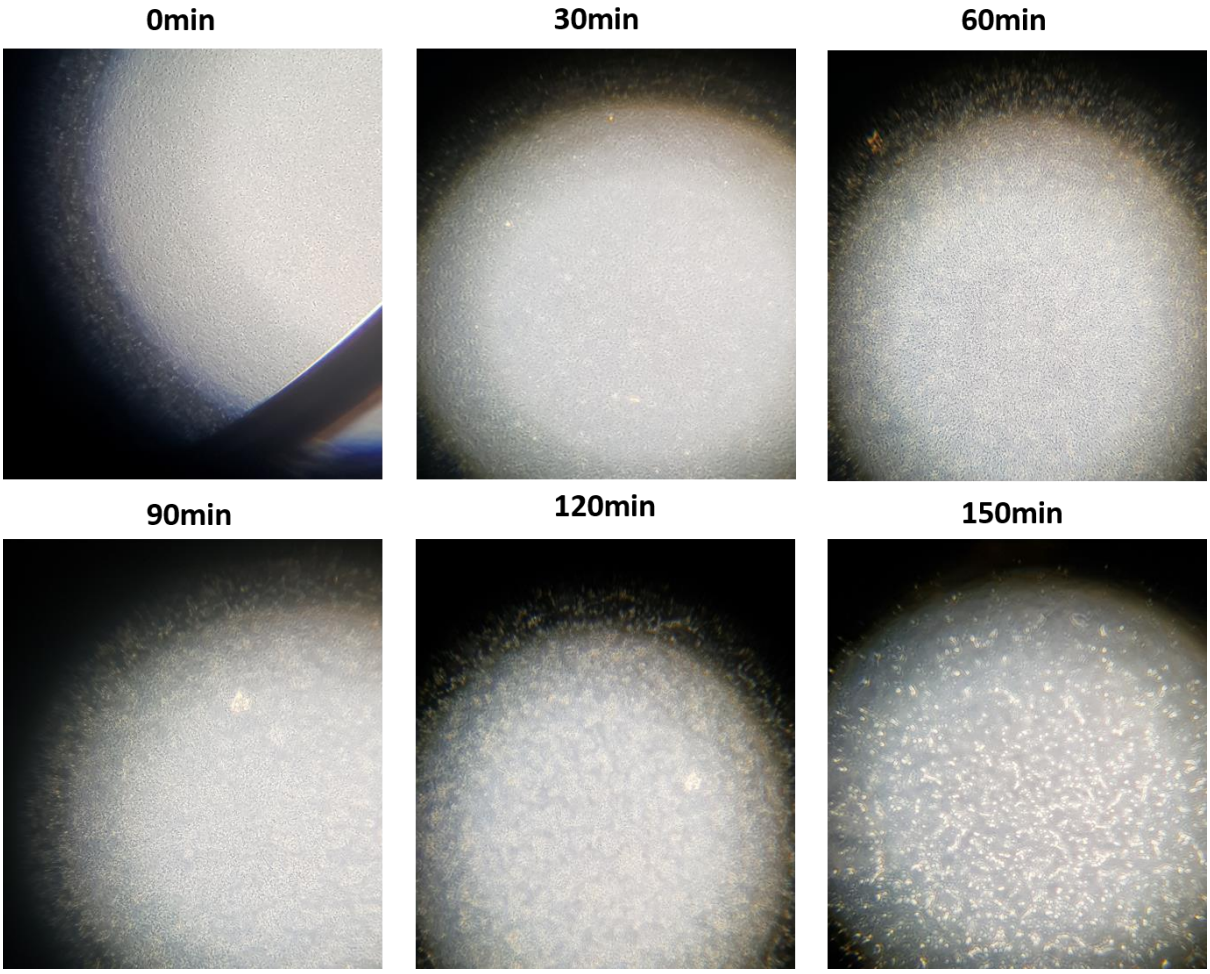

Figure 24: Effects overtime of phase separation induced by H4pep. H4pep at 100uM incubated at room temperature with 0.1ug of DNA Widom 167bp. At the t0 its possible to see only precipitated that further transforms in to gel like phase separation.

The results suggest that at high concentrations the H4pep can exit the precipitated state, seen as small black dots under the microscope and form a hydrogel like structure over time, despite the viscosity was not measure, its possible to speculate, due to the lack of fluidity observed, and the lack of fusion and fission events.

17.3. Salt-resistance assay

The phase separation phenomenon happen due to interactions between the multivalent surfaces, changing the ionic strength of the medium can disrupt these interactions, resulting in the disassemble of the LLPS and for gel or solids phase separations remains partially dissolved (Alberti, Saha et al. 2018, Wang, Zhang et al. 2018). Therefore, I challenged the solution of H4pep and DNA to different salt concentrations in order to characterize the physical state of the phase separation, see Figure 25.

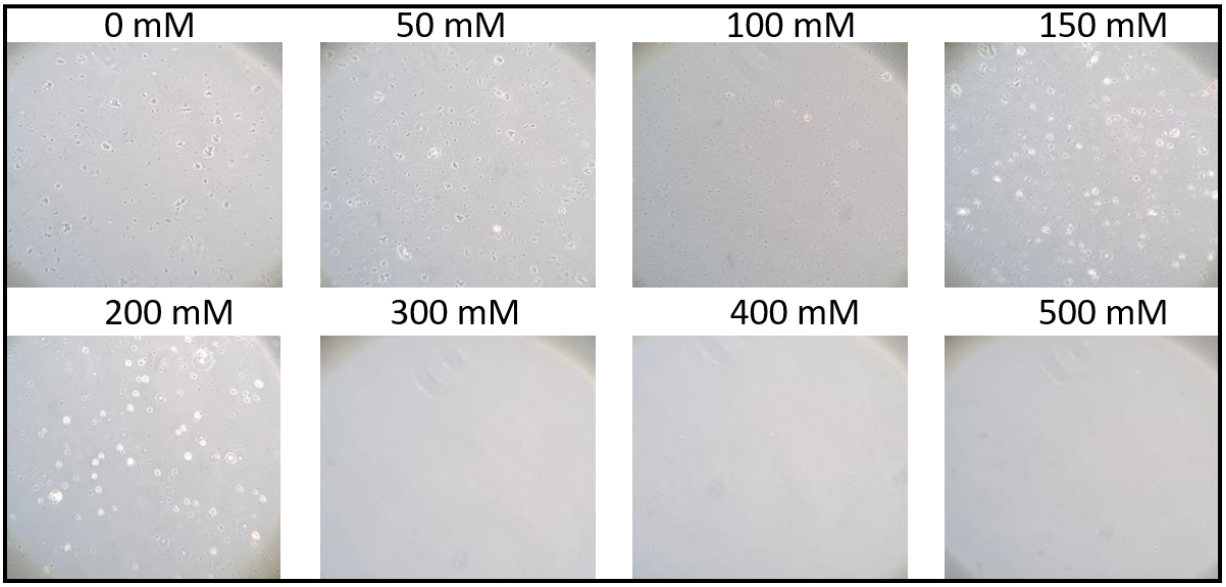

Figure 25: Salt-resistance assay of H4pep whit DNA. Increments in NaCl concentration cause precipitates to form round droplets around physiological salt concentrations, at higher salt concentrations the droplets disappear, suggesting the formation of LLPS. Images are representative of three separate experiments.

It's possible to observe that at low concentrations of NaCl (from 0 to 100) the formation of precipitates happen, when increasing salt concentration tend to diminish, at 150mM of NaCl (physiological conditions) it is also observed the formation of round droplets and the disappearance of the black dots that are the results of precipitation, at 200mM of NaCl droplets show greater roundness, a characteristic of LLPS, when the salt concentration reaches 300mM, no droplets nor

precipitated are observed. This result suggested that the phase separation of H4pep + DNA is liquid. However, experiments such as FRAP are necessary to confirm, along with events of fusion and fission events that ware not observed.

The full characterization of the events related to phase separation requires several others techniques that were not performed in this work, it is not clear in what state the phase separation of H4pep+DNA if is a LLPS or the formation of hydro-gels, despite the salt resistance assays strongly suggesting that is liquid, the droplets did not show any fluidity, also, it was tested only one synthetic DNA sequence, it would be interesting to evaluate different sequences and kinds of DNA. The experiments were not done with the nucleosome as well, investigate if H4pep can induce phase separation at the nucleosomal and/or chromatin level would yield important data. Furthermore, since the H4pep is a histone binding motif, it would be interesting to verify the effects of acetylation in this processes, and also to conduct analysis in others proteins that have histone binding motifs such as HsfB1, Sall1-4, DNMT1 and especially p53, due to its critical role in cancer progression.

18. CONCLUSION

The data presented here suggest that the binding of H4pep to DNA induce phase separation under physiological conditions *in vitro*. Further studies will be important to clarify the phenomenon *in vivo* and whether may impact chromatin dynamics and transcriptional outcome. Also, it will be important to verify whether the H4pep interaction to the nucleosomal DNA in the context of chromatin may induce the formation of phase separation *in vivo* and *in vitro.*

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20. APPENDIX A

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Nucleosome binding peptide presents laudable biophysical and *in vivo* effects

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ABSTRACT

Chromatin state is highly dependent on the nucleosome binding proteins. Herein, we used a multipronged approach employing biophysical and *in vivo* experiments to characterize the effects of Nucleosome Binding Peptides (NBPeps) on nucleosome and cell activity. We performed a series of structure-based calculations on the nucleosome surface interaction with GMIP1 (a novel NBPep generated in silico), and HMGN2 (nucleosome binding motif of HMGN2), which contains sites that bind DNA and the acid patch, and also LANA and H4pep (nucleosome binding motif of H4 histone tail) that only bind to the acidic patch. Biochemical assays shows that H4pep, but not HMGN2, GMIP1 and LANA, is highly specific for targeting the nucleosome, with important effects on the final nucleosome structure and robust *in vivo* effects. These findings suggest that NBPeps might have important therapeutic implications and relevance as tools for chromatin investigation.

1. Introduction

Chromatin is a macromolecular complex composed of distinct molecules. Highly basic proteins (histone octamer) interact with DNA to form the nucleosome core particle (NCP), generating the fundamental repetitive unit of chromatin. The NCP represents the first level of DNA compaction, followed by a cooperative nucleosome interaction to form the higher-order chromatin structure (reviewed in [1]). Chromatin dynamics, which is controlled by a plethora of Nucleosome Binding Proteins (NBPs), is essential for genome integrity and gene expression regulation. From condensed to relaxed chromatin, NBPs may induce specific modifications of chromatin architecture dependent on their unique properties [2]. In addition to NBPs, the nuclear environment also comprises many small molecules with different chemical natures that can directly interact with nucleosomes, such as Mg^{2+} and lipids [3,4].

The nucleosome core particle surface contains the acidic patch, a highly negative region formed by six residues of H2A and two of H2B, that is responsible for nucleosome–nucleosome interactions and is a target for several NBPs [2]. The first structure of a nucleosome:peptide complex showed at atomic level the binding mode of the viral peptide LANA to the acidic patch [5]. In the following years, other nucleosome:NBPs complex structures were solved, revealing the atomic details of the interaction of NBPs with the nucleosome surface, highlighting the acidic patch as the principal protein-docking region [6].

Unlike canonical drug targets such as enzymes or protein receptors, the nucleosome is a structural protein:DNA complex without typical druggable cavities, and this impedes research for new exogenous nucleosome binding molecules. Instead of focusing on small molecules for occupying the nucleosome surface, we try to understand and developed more complex molecules, such as the Nucleosome Binding Peptides (NBPeps).

Herein, we characterized the effects of NBPeps on nucleosome and chromatin structure. Firstly, we designed and generated *in silico* a novel NBPep, GMIP1, with nucleosome surface binding highly dependent on the DNA. Then, in order to understand how NBPeps with distinct nucleosome binding sites affect nucleosome structure, we performed a series of structure-based calculations on the nucleosome surface interaction to the NBPeps. We studied four NBPeps, GMIP1 and HMGN2 (the nucleosome binding motif from HMGN2), which contains sites that

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bind DNA and the acid patch, and also LANA and H4pep (structure-based nucleosome binding motif derived from the N-terminal domain of histone H4) that only bind to the acidic patch. Interestingly, we observed that NBPeps induced specific atomic fluctuations of the nucleosome structure. *In vitro* studies corroborate the idea that NBPeps may affect the stability of the nucleosome structure, however only H4pep showed high nucleosome binding affinity and specific actions on the final nucleosome structure while HMGN2, LANA and GMIP1 peps seems to be non-specific DNA interactors, based on oppositely charged residues. Cell-based assays showed that the four NBPeps penetrated the cell, localizing at the nuclear environment and, except GMIP1, interfered with tumor cell viability. However, beside the non specific properties on the nucleosome, apart from H4pep, the fish embryo toxicity (FET) test showed that the four NBPeps could cause tissue modifications, such as defects in pigmentation and induction of earlier hatching.

2. Methods

2.1. In silico NBPep design

KV finder software with a PyMOL interface plugin (The PyMOL – Molecular Graphics System Version 1.3 Schrodinger, LLC) was used to define the cavities on the nucleosome:protein complex. YSARA software was used for the GMIP1 design and the optimized conformation by minimizing energy was performed using the force field YAMBER3.

2.2. NBPeps

All Peptides were bought from Biomatik with purity > 95 % and diluted in MiliQ H_2O. Fluorescent peptides were bought with TAMRA -(559/583 nm) in the N-terminus. The concentration was determined by spectrophotometric method as described in [7]. All peptides are described in the supplementary table 1.

2.3. Recombinant H2A-H2B for NMR

BL21(DE3)pLysS cells were used to express *Xenopus laevis* H2A or H2B in deuterated M9 medium containing 2 g/l (12C-2D) D-glucose (1,2,3,4,5,6,6-d7) and 0.5 g/L 15NHCl. One hour before induction with 1 mM IPTG, 60 mg/L α-ketobutyric acid (4–13C,3,3-2d) and 80 mg/L α-ketoisovalerate (3-(methyl-d3),4–13 C,3-d) sodium salt were added to the medium. Histones were purified as described by Dyer et al. [8] To refold dimers, H2A and H2B were mixed in equimolar ratio in unfolding buffer (7 M urea, 150 mM NaCl, 50 mM NaPi, 1 mM EDTA, pH 7.5) and dialysed against high salt buffer (2 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5). Dimers were purified using size exclusion chromatography (Hiload superdex 200 16/600) and then dialysed against low salt buffer (20 mM NaPi, 0.01 % NaN_3, pH6.2).

2.4. In vitro chromatin fibers and nucleosome reconstitution

Histone octamers (HO) were purified from chicken erythrocyte nuclei as described in Huynh, V. A. T., P. J. J. Robinson, and D. Rhodes, 2005. 601 DNA Widom with 167 base pairs (bp) was used to reconstitute mononucleosomes and array 177.36 was used to reconstitute 10 nm chromatin fibers, both using the slow salt dialysis method as described in Huynh et al. (2005) [9]. The analyses of the reconstitution were verified by electrophoresis in native bis-acrylamide gels (6 %) or agarose gels (0.8 %).

2.5. Mononucleosome precipitation

Freshly reconstituted mononucleosomes (115 nM mononucleosome, Tris 10 mM pH 7.4, EDTA 1.5 mM NaCl 15 mM) were incubated with specified concentration of NBPeps for 30 min at room temperature. The samples were centrifuged (Sigma centrifuge-2K15) at 15,493 x g for 20 min at 25 °C. The supernatant was transferred to another micro-centrifuge tube and the pellet was resuspended in the same buffer as the mononucleosome. The samples were analyzed by electrophoresis in native 6 % bis-acrylamide gel carried out with 0.5 × TBE buffer at 15 mA. Densitometry was performed using ImageJ (National Institute of Health, Bethesda, MD, USA) version 1.49.

2.6. DNA binding assay

Widom 601 DNA fragments containing 167bp (30 nM DNA, 10 mM Tris pH 7.4, 135 mM NaCl) were incubated with specified concentrations of GMIP1 for 2 h at 37 °C and 100 RPM. The analysis was done in 0.8 % agarose gel in TBE 0.5 × . Samples were loaded with 30 % glycerol, to avoid interaction caused by phenol blue and GMIP1.

2.7. Nucleosome binding assay

Freshly reconstituted mononucleosomes (115 nM mononucleosome, Tris 10 mM pH 7.4, EDTA 1.5 mM NaCl 15 mM) were incubated with specified concentration of fluorescent NBPeps for 120 min at room temperature. Then samples were analyzed by electrophoresis in native 6 % bis-acrylamide gel carried out with 0.5 × TBE buffer at 15 mA. Gels were analyzed using Amersham Imager 600 (GE) with the RGB laser kit detection for 520 nm, to visualize the peptide, following incubation in ethidium bromide bath and analyzed with UV for ethidium bromide detection. For Kd determination, band densitometry was performed in the gel reveled with 520 nm laser, using ImageJ (National Institute of Health, Bethesda, MD, USA) version 1.49, followed by analysis in Prism 6 Graphpad software using Binding - saturation binding to total and non-specific template.

2.8. Chromatin compaction assay

Chromatin compaction by Mg^{2+} was adapted from Rhodes Lab protocol [10]. Briefly, assembled chromatin fibers were incubated with vehicle (10 mM Tris−HCl (pH 7.5) or peptides (150μM GMIP1 or H4pep) for 2 h in room temperature. Next, 3 mM $MgCl_2$ were added, incubated for 15 min on ice and centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatants and pellets were verified by electrophoresis in native 0.8 % agarose gels, carried out with 0.2x TBE (18 mM Tris-borate, pH 8; 0.4 mM EDTA) electrophoresis buffer at 20 mA. Densitometry was performed using ImageJ (National Institute of Health, Bethesda, MD, USA) version 1.49.

2.9. Thermal shift assay

Thermalshift assay with NBPeps was adapted from Taguchi et al. [11]. Briefly, freshly reconstituted mononucleosomes (86 nM), in 10 mM Tris pH 7.4, 1.5 mM EDTA and 15 mM NaCl, were incubated with specified concentration of NBPeps for 30 min at room temperature. Next 1 mM dithiothreitol and 5X of SYPRO-Orange (SIGMA–ALDRICH) were added and incubated for 1 min at room temperature. Fluorescence was measured with a StepOnePlus Real-Time PCR unit (Applied Biosystems) with increases of 1 °C step from 25 °C to 95 °C. The fluorescence was detected at 570 nm. The Raw data was normalized using Graphpad Prism 6 software and for the determination of the temperature of melting (Tm) was obtained from the first derivative curve of the data.

2.10. MTT

For MTT assays, 8000 Hela cells or ccd10595k cells were plated in 96-well culture plates and maintained at 37 °C and 5 % CO_2 in DMEM medium with 10 % fetal bovine serum, penicillin (100U/mL) and streptomycin (100ug/mL) for 24 h. Next, wells were washed 3 times

with PBS 1X and filled with 100 u L of DMEM medium as described above containing the specified amount of NBPeps and incubated for 24 h in the same conditions. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 5 mg/mL was added to the wells (10 u L) and incubated for 4 h at 37◦C and 5 % CO_2. The wells were drained and the formazan crystals were solubilized in 100 u L of acidic isopropanol solution (52 u L of HCl 37 % to 12 mL of isopropanol) and agitated for 30 min at room temperature. Absorbance at 570 nm was determined with a plate spectrophotometer (DTX 800 Multimode Detector - Beckman Coulter) at 570 nm.

2.11. Flow cytometry

70.000 Hela cells were plated in 12-wells culture plates for 16 h and maintained at 37 °C and 5 % CO_2 in DMEM medium with 10 % fetal bovine serum, penicillin (100U/mL) and streptomycin (100ug/mL). Prior to treatment with NBPeps, cells were incubated for 1 h at 37 or 4 °C. Next, cells were washed with 1X PBS and filled with DMEM medium with the specified amount of fluorescent NBPeps and incubated at 37 or 4 °C for the 1 or 3 h. Wells were washed 3 times with ice cold 1X PBS and filled with 500 u L 1X PBS, cells were harvested with a cell scraper and analyzed by flow cytometry on FACSCalibur (BD biosciences). Hela cells were gated to isolate the main population of living cells from cell debris. Data analysis was done using flowjo 8.7 software.

3. Fluorescence confocal microscopy analysis

i) **Peptide cell penetration**: confluent Hela cells were treated with increased concentrations (0,1; 0,5; 1μM) of peptide GMIP1-TAMRA or vehicle (10 mM Tris−HCl) for 24 h. Next, plates were rinsed three times in PBS and fixed in formalin 3.7 % at room temperature for 10 min. Samples were rinsed three times in PBS and incubated with DAPI (300 ng/ml) for 5 min to stain nuclei. Finally, cells were rinsed three more times in PBS. Images were acquired using a laser scanning confocal microscope Leica TCS SP5. To visualize peptide localization, Z-planes of 0.20 mm thickness were acquired. The images were analyzed with LAS AF software (Leica Microsystems CMS GmbH).

ii) **Determination of peptide internalization**: two plates of 24 wells with confluent Hela cells were rinsed with DMEM without fetal bovine serum twice and maintained in PBS for 15 min in a 5 % CO2 humidified atmosphere at 37 °C. One plate was then maintained for 10 min at 4 °C. Next, the two plates were treated with GMIP1-TAMRA (1μM) of vehicle for 5, 15 and 30 min. After each treatment samples were fixed in formalin 3.7 % at room temperature for 1 h. Samples were rinsed three times in PBS and incubated with DAPI (300 ng/ml) for 5 min to stain nuclei. Finally, cells were rinsed three more times in PBS. Images were acquired using a laser scanning confocal microscope Leica TCS SP5. To visualize peptide localization, Z-planes of 0.20 mm thickness were acquired. The software LAS AF (Leica Microsystems CMS GmbH) was used for analyse images.

3.1. Zebrafish husbandry and embryo collection

Zebrafish *(Danio rerio)* were raised in an aquatic facility (ZebTec - Tecniplast, Italy) with a photoperiod cycle of 12:12 h (light:dark) at the University of Brasilia (Brazil). The water parameters were: temperature was maintained at 27.0 ± 1 °C, conductivity at 650 ± 100 μS/cm, pH at 7.0 ± 0.5 and dissolved oxygen ≥95 % saturation. Zebrafish embryos were collected immediately after natural mating, rinsed in water, and checked under a stereomicroscope (Stereoscopic Zoom Microscope – Stemi 2000, Zeiss, Germany). The unfertilized eggs and those showing cleavage irregularities or injuries were discarded [12].

3.2. Fish embryo toxicity (FET)

FET was adapted from Morash et al. [13]. Briefly, Zebrafish embryos at 4, 28 and 52 h post fertilization (hpf) were used to evaluate the toxicity of NBPeps in 96-well plates. Each peptide was tested at 0.1, 1, 10 and 100 u M in 100 u L of water from aquarium system; pH in all conditions was tested using pH strips (92,120 – MACHEREY-NAGEL). Embryos were stored at 27 °C with 14 h light 10 h dark cycle and evaluated Stemi 508 (Carl Zeiss) microscope with 1 and 24 h of treatment. Embryos were assessed for pigmentation, development, hatching and lethality. 10 embryos were used for each condition, if the control group showed any alteration, the plate was discarded, alterations > 10 % were considered significant and were documented using Axiocam Erc 5 s (Carl Zeiss) and ZEN software (Carl Zeiss).

3.3. Fluorescence fish embryo

Zebrafish larvae with 80 hpf were incubated with fluorescent NBPeps with specified concentration for 3 h in 100 u L in a 96-plate, larvae were washed 3 times in 100 mL to remove the excess of NBPep, imaging was done using Axioskop 2 (Carl Zeiss) with HBO 100 lamps, Axiocam Erc 5 s (Carl Zeiss) and ZEN software (Carl Zeiss) with appropriate laser filter for TAMRA (filter 4).

3.4. Fluorescence blood smear

Adults Zebrafish at 2 years old were inject in the abdomen with 50 u L, 1 mM of fluorescent NBPeps, and kept protected from light at 27 °C for 18 h. Blood was extracted from the fins using a pipet tip and heparin 250 IU to make the blood smear in a microscope slide. Images were acquired with Axioskop 2 (Carl Zeiss) with HBO 100 lamps, Axiocam Erc 5 s (Carl Zeiss) and ZEN software (Carl Zeiss) with appropriate laser filter fom TAMRA (filter 4).

3.5. NMR

All NMR experiments were carried out on a Bruker advance III HD 600 MHz. NMR spectra were processed in Bruker TopSpin [14] and analyzed using Sparky [15]. Dimer samples of [C^{13},N^{15}]H2A-H2B at 100 u M in 5 %D_2O/95 %H_2O; 25 mM NaPi + 100 mM NaCl pH6.2 + 0,01 % NaN3 + 1 mM 2-Mercaptoethano + PIC (complete EDTA-free Protease Inhibitor Cocktail (Roche)) were titrated against GMIP1 using 600 MHz Lamour frequency at 308 K. HSQC spectra were measured for free [C^{13},N^{15}]H2A-H2B and after the addition of GMIP1 at 308 K. Titration consisting of 4 points in the range of 1:4.3 M ratio ([C^{13},N^{15}]H2A-H2B:GMIP1) was performed.

3.6. Circular dichroism

Measurement of secondary structure of NBPeps was performed in Jasco *j*-815 spectropolarimeter in a 0,1 cm quartz cuvette in the range of 190−250 nm. Samples were diluted in MiliQ water in the concentration of 0.125 mg/mL for GMIP1, LANA, HMGN2 and H4pep at 0.107 mg/mL at 25 ◦C. Data were plotted using BestSel data base (available at: http://bestsel.elte.hu/).

3.7. Computational studies NBPeps

Nucleosome Atomistic models were built using a high-resolution x-ray structure of the nucleosome (NCP) without histone tails, PDB code 3TU4 [16]. Peptides were simulated free in solution or in association with nucleosome at 1:1 and 2:1 stoichiometries. Table M&M provides information on the construction and initial atomic coordinates for the various systems under investigation. Analyses were performed with VMD version 1.9.3 [17].

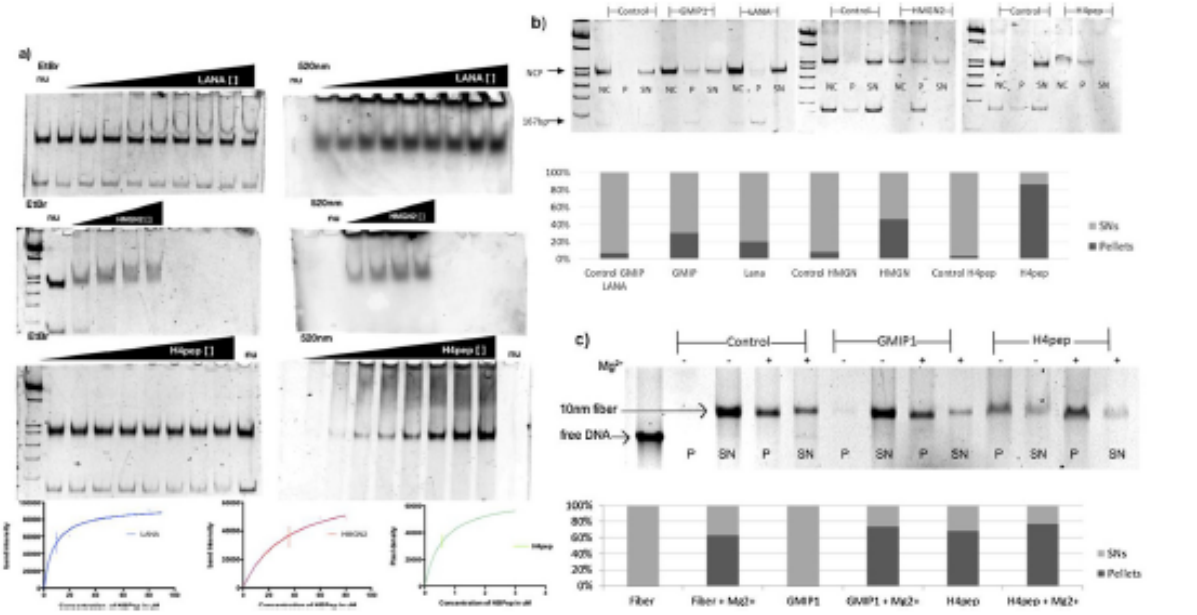

Fig. 1. NBPeps interaction assay a) Nucleosome binding assay with fluorescent NBPeps, nucleosome is incubated with LANA at 0, 10, 20, 30, 40, 50 60, 70 80, 90 u M, with HMGN2pep at 0, 20, 40, 60, 80 u M or with H4pep at 0, 200, 400, 600, 800, 1000, 1200, 1400, 1600 nM. It was then analyzed in acrylamide gel, following by detection of the fluorescent NBPep and subsequently detection of DNA. Kd is represented by a vertical line in the densitometry graphs. b) Nucleosome precipitation assay with NBPeps, centrifuged nucleosome without NBPeps stays in the supernatant (SN); the addition of 50 u M GMIP1, 10 u M LANA, 10 u M HMGN2 or 500 nM H4pep induce precipitation and pellet (P) formation, non-centrifuged (NC) samples were used as control. DNA band densitometry graph of upper gel c) Chromatin compaction assay with Mg2 +, 150 u M H4pep induces 36-mer chromatin precipitation, but not 150 u M GMIP1. DNA band densitometry graph of upper gel. DNA band densitometry graph of upper gel. These assays were performed at least 3 times, and the representative gel was presented.

3.8. Molecular docking

AutoDock Vina was used to resolve peptide binding to the acidic patch region of NCP [18]. To account for a larger ensemble of binding modes 20 independent structures randomly collected from the peptide-free MD equilibration was docked to the NCP. The exhaustiveness value was set to 200 and best solutions were gathered from each docking calculation, resulting in approximately 400 solutions per peptide. Solutions were clustered in 15–17 structural groups based on a maximum neighborhood criterion and the group with best fit to the acidic patch was chosen for further simulation.

3.9. Molecular dynamics simulations

The VMD software was used to solvate and neutralize with counter ions the NCP-peptides systems [17], resulting in simulations cells averaging ~ 110 Å x 145 Å x 145 Å with ~ 210.000 atoms and 150 mM sodium chloride. All systems were simulated in a NPT ensemble at 300 K, 1 atm and with 2 fs time step for 115 ns with periodic boundary conditions (PBC) and enough water to avoid any interactions between the PBC images. Each system was thermalized and subsequently equilibrated for ~ 10 ns MD simulation. All simulations were run by NAMD version 2.10 [19] with CHARMM 36 force field [20] and TIP3 water model [21]. PME method [22] was employed on the electrostatic calculations and non-bonded interactions were cut-off at 11 Å. No bias was needed to keep the peptides bound to NCP throughout the entire simulation.

4. Results

4.1. In silico development and analysis of structure-based nucleosome binding peptide

To design and develop a NBPep that best-fitted on the nucleosome surface, we studied available nucleosome binding proteins structures as templates [5,16,23–25]. We used the software KvFinder to identify putative binding pockets and shallow crevices on the nucleosome surface to use these cavities as potential binding site to plan NBPeps [26]. We selected three crystallographic atomic structures to start our search, those of RCC1, Sir3 and LANA. The best-fitted molecule on the acidic patch was RCC1 (Supplementary Fig. 1a), which contains binding sites for both the acidic patch and the associated nucleosomal DNA [23]. Besides the deep anchor on the acidic patch, through R216 and R223, there is another region distal to the acid patch in which the residue T238 from the RCC1 may interact to T75 from H2A. Therefore, based on the structure of RCC1:Nucleosome complex, we generated the new NBPep, GMIP1 (genetic modified inducible peptide 1), which included the two nucleosome binding sites to either DNA or the acidic patch, linked by three alanine residues (Supplementary Fig. 1 b–d).

4.2. Differential binding of NBPeps to the nucleosome and effect on chromatin conformation

We selected NBPeps based on their abilities to bind to the (i) acidic patch (LANA and H4) or (ii) both DNA and acidic patch (GMIP1 and HMGN2).

We already knew that the NBPeps LANA, H4 tail and HMGN2 could bind to the nucleosome [5,24,25], but we did not have biochemical information about GMIP1. Although GMIP1 was based on the nucleosome binding motif of RCC1, it has a new structure, with two independent binding regions linked by three alananine residues as

discussed earlier. Circular dichroism analysis of the NBPeps showed predominant random coil structure (Supplementary Fig. 2 and Supp. table 1 sequence NBPeps). NMR experiments with free GMIP1 indicated the peptide is in a random-coil state based on absence of medium or long-range NOEs and random coil 13C chemical shifts (Santos GM and van Ingen H., personal communication).

To observe the binding and the impact of NBPeps to the nucleosome we performed nucleosome binding and precipitation assays (Fig. 1 a). First, we reconstituted nucleosomes *in vitro* with histone octamer from chicken and 167.1 (167 base pairs) DNA 601 and incubated it with labelled NBPeps-TAMRA. It demonstrated that H4pep binds to the mononucleosome at a Kd of 0,6 u M, LANA at Kd of 8 u M and HMGN2 at 358 u M respectively. GMIP1 induced nucleosome aggregation even in low concentration, which difficulted to determine the binding affinity constant. HMGN2pep promotes an electrophorectic mobility shift, suggesting that it is binding at more than one site on the nucleosome surface, further investigations will be need to explore this finding.

To confirm the binding assays results, non-labelled GMIP1, LANA, HMGN2 and H4pep were then incubated with mononucleosomes and then centrifuged. We observed that the nucleosome in absence of NBPeps does not precipitate, but all four NBPeps variably induced nucleosome precipitation since part of the complex was found in the pellet (Fig. 1 b). Notable, H4pep induced precipitation at 500 nM. At higher concentration, the NBPeps induce mononucleosome aggregation and did not migrate in the gel (data not shown).

To understand and validate the binding epitope of GMIP1 on the histone H2A:H2B surface, we performed an interaction study with *in vitro* reconstituted and isotope-labeled *xenopus laevis* H2A/H2B dimers by NMR. Binding of unlabeled GMIP1 should result in specific and clear changes in either peak position and/or intensities. Surprisingly, no significant spectral changes were observed even at high excess of GMIP1 (Supplementary Fig. 1 e), suggesting that this new NBPep needs DNA binding to be stable on the nucleosome. Indeed, GMIP1 bound to the naked DNA *in vitro* (Supplementary Fig. 1 f) but at a Kd of 50μM or weaker, which is probably in the range of non-specific binding of a charged peptide to DNA. Furthermore, GMIP1 showed to induce nucleosome aggregation which difficulted the Kd determination. Nucleosome (Supplementary Fig. 1 g). We then applied docking and Molecular Dynamics simulations to study binding of the GMIP1 at the atomic level and the conformational stability of the nucleosome. It showed that H2A/H2B dimer is not sufficient to hold GMIP1 on the nucleosome. Interestingly, the nucleosomal DNA stabilizes GMIP1 on both DNA and H2A/H2B dimer (Supplementary video and Supplementary Fig. 3).

It is already known that the non-acetylated H4 tail is able to induce chromatin condensation. [27].In order to check whether GMIP1, which low affinity nucleosome binding is similar to LANA and HMGN2pep, could also affect the chromatin state such as described for non-acetylated H4 tail, we performed a chromatin compaction assay with Mg^{2+} (Fig. 1 c). For this, we reconstituted long chromatin fibers, 177.36 (36 nucleosomes), *in vitro* and incubated them with H4pep and GMIP1 and then with Mg^{2+}, following to centrifugation. Since the precipitated chromatin fiber recovered from the pellet was not cross-linked, it did not hold the compacted state and migrated in the gel similarly to the relaxed chromatin fiber, as observed in the gel. H4pep induced precipitation even in absence of Mg^{2+}, and precipitation increased when the divalent cation was added. However, GMIP1 did not show any effect alone, even when it was in presence of Mg^{2+}.

Taguchi et al. have established a technique to evaluate the physical properties of nucleosomes, developing a convenient assay of the thermal stability of nucleosomes *in vitro*. It was observed that increasing temperature induces nucleosome denaturation in two steps, firstly at 75 ℃ the eviction of H2A:H2B dimers occurs followed by the eviction of H3:H4 tetramers at and at 85 ℃ [11]. Therefore, herein thermal shift assays were performed to further check the hypothesis that NBPeps affect nucleosome stability (Fig. 2). GMIP1 showed a dose dependent effect of the nucleosome stability, affecting first the H2A:H2B dimers, with a pronounced effect at higher concentrations, 150 u M. LANA has a notable effect on the H3:H4 tetramers, starting at 30 u M. HMGN2 induced earlier disruption of the H3:H4 tetramers at 3u M. Clearly, H4pep induced H2A:H2B dimer stabilization at 100 nM concentration and did not affect H3:H4 tetramer. These findings suggested that NBPeps affect the nucleosome stability in different manners, however only H4pep presented a high specificity effect.

4.3. The interaction mode of NBPeps and nucleosome

In order to understand how the nucleosome structure senses the binding of NBPeps, MD simulations were performed to study the NBPeps based on their abilities to bind to the acidic patch or both DNA and acidic patch. More specifically, we studied peptide motifs from LANA and H4 tail that just bind to the H2A and H2B of the acidic patch and, for the second group, the peptide motif from HMGN2 and the new GMIP1 that bind to H2A and H2B and DNA. A scrambled peptide derived from GMIP1 was used as a negative control. All NBPeps tested here, but not the negative control, were able to bind and maintain the interaction with nucleosome over 115 ns of equilibrium trajectory (Fig. 3a).

It was observed that NBPeps induced specific atomic fluctuations of the nucleosome structure. Based on the differences of RSMF (root square mean fluctuation) of the nucleosomes, in presence and absence of the NBPeps, a map of the fluctuations was generated. There were several common fluctuation changes caused by the different NBPeps, however it is important to highlight the fluctuations of two distinct regions of the nucleosome (circles and arrows Fig. 3b). First, NBPeps that interact with just the acidic patch caused higher atomic movement at the nucleosome entryside-DNA region (arrows Fig. 3b). LANA significantly increase the DNA atomic fluctuations and H4pep slightly increase fluctuations of the H3 N-terminal helix. As expected, GMIP1, but not HMGN2, which also bind to the DNA, moderately reduced the DNA fluctuation. The second region affect by NBPeps binding was one of the known interactions points between the DNA and histones [25] (circles Fig. 3b). This interface, composed by H4 amino acids A76, K77, R78 and 8 nucleotides, showed narrow movement reduction caused by all NBPeps. Also, although rather small, HMGN2 was the peptide with greater impact (increasing) on the histones octamer atomic fluctuations.

Changes in hydration pattern of the nucleosome showed that the NBPeps reduced at least water content by 10 % on the acidic patch. GMIP1 removed fewer water molecules compared to the other NBPep, even when compared to HMGN2 that also has two binding sites, DNA and acidic patch (Fig. 3c).

4.4. NBPeps cellular uptake and viability

Cell penetrating peptides, CPPs, are typically short peptides composed by high content of positively charged amino acids, such as lysine and arginine. Interestingly, NBPeps also contain several positively charged aminoacids [28]. In order to verify whether the four NBPeps could penetrate the cell environment, we performed flow cytometry assays in Hela cells. The four NBPeps penetrated the cell at 37 ℃ and GMIP1 had the highest uptake. H4pep in higher concentration (10 u M) was also taken up at 4 ℃ at a similarly rate to 37 ℃ (Fig. 4a).

To better understand and characterize whether GMIP1 that presented low affinity nucleosome binding could be uptaken and bind to the chromatin, we treated Hela cells with labeled GMIP1 and analyzed them with confocal microscopy. Images with orthogonal views demonstrated that peptide GMIP1 penetrates the cells even in the smallest concentration (0.1μM). Confocal microscopy merged images of labeled GMIP1 and DNA stained with DAPI indicate the presence of GMIP1 associated with chromatin (Fig. 4b). Internalization of GMIP1 in Hela cells occurred only at 37 ℃, suggesting an endocytosis transport

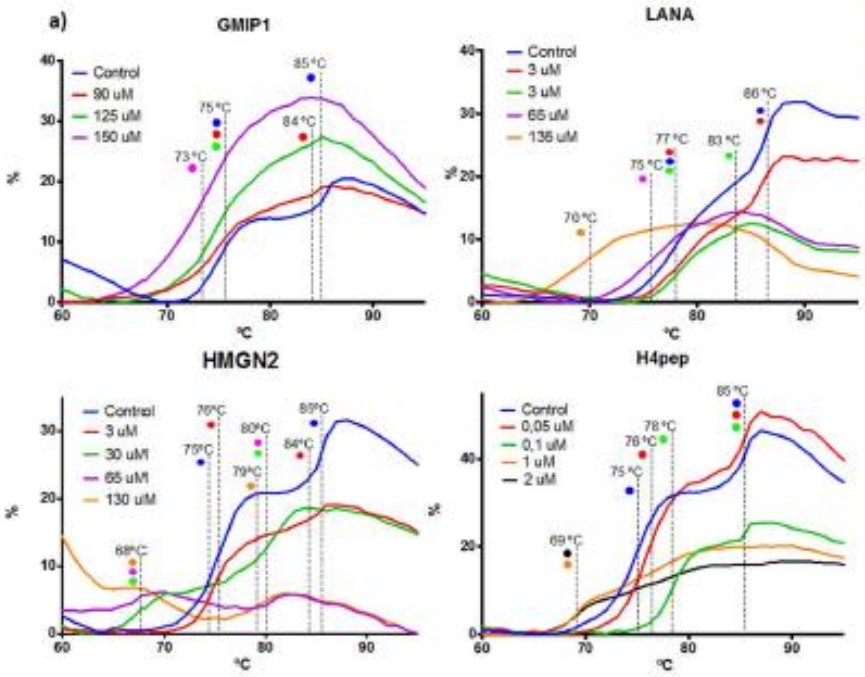

Fig. 2. Thermal Shift assay a) Thermal denaturation profiles of mononucleosomes in the presence of NBPeps. Mononucleosome denaturation happens in two steps, the first peak is the eviction of the dimers H2A/H2B and the second step if the eviction of the tetramers H3/H4 the formation of a peak at 68 – 70 °C appears when the mononucleosome is already disassembled. All thermal denaturation profiles are representative of three separate experiments.

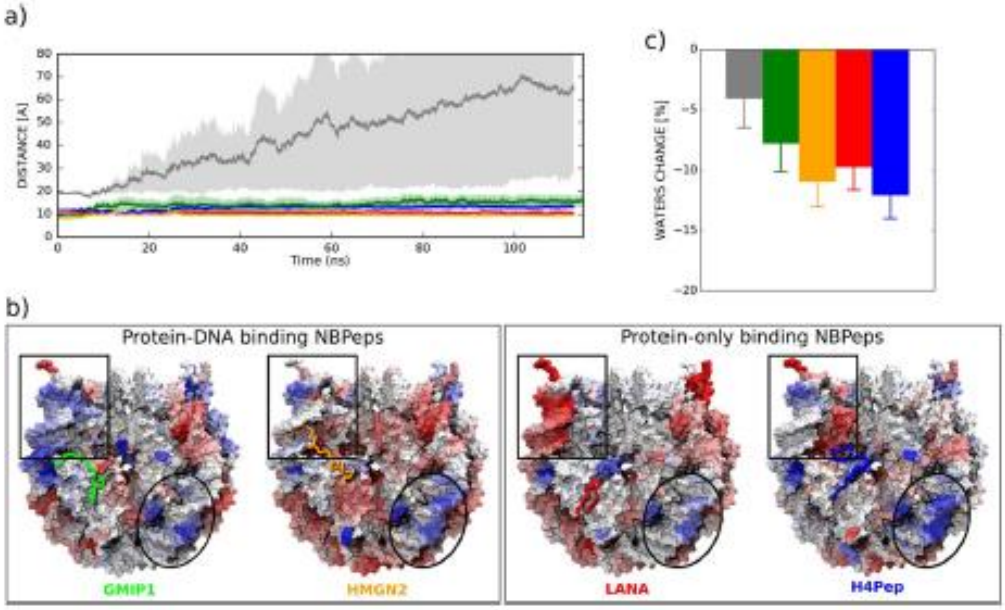

Fig. 3. NBPeps interactions with the nucleosome a) Time-dependent centroid distances profiles between the acidic patch region and NBPeps (Negative control – gray; GMIP1- green; HMGN2 – orange; LANA – red; H4pep - blue). Shaded regions indicate standard error between all simulations. b) NBPeps-induced proportional changes on the nucleosome (NCP) root-mean-square fluctuations (RMSF). The ratio was calculated after averaging all peptides association with the nucleosome stoichiometries. Color scale values distinguish between higher (red) and lower (blue) fluctuations compared to the RMSF of NBPep free nucleosome, with a 25 % cut-off. Squares and circles indicate strongest fluctuation changes regions c) Average of proportional change in the number of water molecules occupying the acidic patch for each NBPep. The change was calculated using the nucleosome free simulation as base value and within a cut-off distance of 20 Å. Averages and associated standard deviation were computed from 100 ns simulation. Colors code as a).

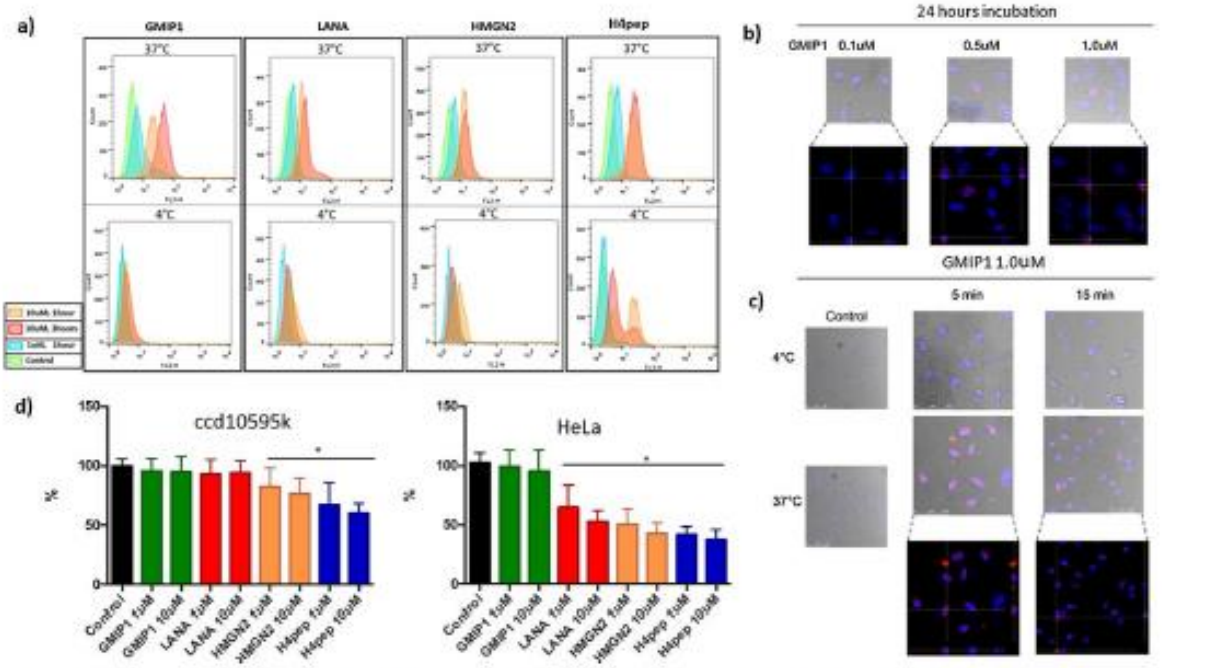

Fig. 4. NBPeps cell penetration a) Flow cytometry profile of Hela cells uptake of fluorescent NBPeps (TAMRA), in histogram view with 1 or 3 h exposure and at 37 or 4 °C. Histogram is representative of three separate experiments.
b) GMIP1 bound to chromatin: confluent Hela cells were treated with increased concentrations (0.1; 0.5; 1 μM) of peptide GMIP1-TAMRA or vehicle (10 mM Tris – HCl) for 24 h. The images were acquired using a laser scanning confocal microscope Leica TCS SP5. C) GMIP1 internalization: Ortogonal analysis of GMIP1 overlapping with stained (DAPI) DNA. Cell penetration only occurs at 37 °C. The images were acquired using a laser scanning confocal microscope Leica TCS SP5. To visualize peptide localization, Z-planes of 0.20 μm thickness were acquired. The software LAS AF (Leica Microsystems CMS GmbH) was used for analyze images. All images are representative of three separate experiments. d) Relative cell viability compared to control over 24 h exposure to NBPeps in HeLa and CCD 10,595 K. HeLa cells showed a greater decreased in cell viability than CCD 10595k for every NBPep tested with the exception of GMIP1. Data is shown as mean ± SD. * represent significant statistical difference (one-way ANOVA test) between the control and treated groups with = $p < 0.05$ and $n = 2$.

(Fig. 4c).

4.5. Cell viability assay with NBPeps

In order to verify the potentiality of NBPeps for modulating cell physiology, we performed MTT assay using with different cell linages. In general, NBPeps, but not GMIP1, significantly affected the viability of Hela cells, which support the evidences obtained *in vitro* that GMIP1 is acting non-specifically. Furthermore, NBPeps impacted the primary cell lineage ccd10595k much less, except H4pep that still showed high effect on the cell viability (Fig. 4d).

5. Fish embryo toxicity (FET) with NBPeps

To analyze *in vivo* the action of the NBPeps, we performed we performed Fish Embryo Toxicity (FET, OECD protocol n.236, 2013) test. After the exposure to NBPeps, at 3 different stages of development, at 4, 28 and 52 h post-fertilization (hpf) for 1 and 24 h, all NBPeps presented low or no toxicity (Supplementary table 2).

We were able to identify the labeled NBPeps in the nuclei of adult zebrafish erythrocyte (Fig. 5a). Moreover, NBPeps penetrated different tissues of fish larvae, after 3 hs incubation (Fig. 5b).

The respective outcome of the embryo's exposure to the NBPeps was highly dependent on the stage of development (Fig. 5c). The hatching rate of the zebra fish was largely affected by the presence of the NBPeps, with GMIP1 showing the strongest effect (Supplementary table 2).

GMIP1 presented a discrete pigmentation defect, in 26 % of embryos when at 28hpf and did not induce mortality at any concentration tested.

LANA, at the highest concentration (100 u M), caused delay in the development of 100 % of the embryos when they were exposed at 4hpf. Also, LANA induced an increased hatching rate (Fig. 5c and Supp. table 2).

HMGN2 clearly affected pigment formation of 16 % of the embryos at 100 u M when at 56hpf.

Remarkably, H4pep were the only NBPeps tested that induced 100 % mortality at 4hpf, and the exposure for 1 h induce 20 % of mortality.

6. Discussion

Proteins that bind the nucleosome, the repetitive unit of chromatin, and the histone H4 tail are critical for establishing chromatin architecture and phenotypic outcomes [29]. A myriad of histone modifications, which strongly induce changes in chromatin structure, have been associated with malignancies and other diseases, highlighting the crucial role of chromatin architectural changes in disease mechanisms [30]. Thus, it is plausible that any nucleosome binding molecule might be able to interfere with the chromatin dynamics and modulate the access of proteins to the DNA.

At first glance, small molecules would be the best option for engaging the nucleosome surface, mainly the acidic patch, to control chromatin dynamics. An effort to identify specific inhibitors of Kaposi's sarcoma-associated herpesvirus (KSHV) focused on small molecules that could displace the LANA peptide from the acidic patch [31]. Investigators tested more than 350.000 small molecules, but could not find any compound able to displace LANA from the nucleosome surface, suggesting that more complex molecules, such as peptides, might be

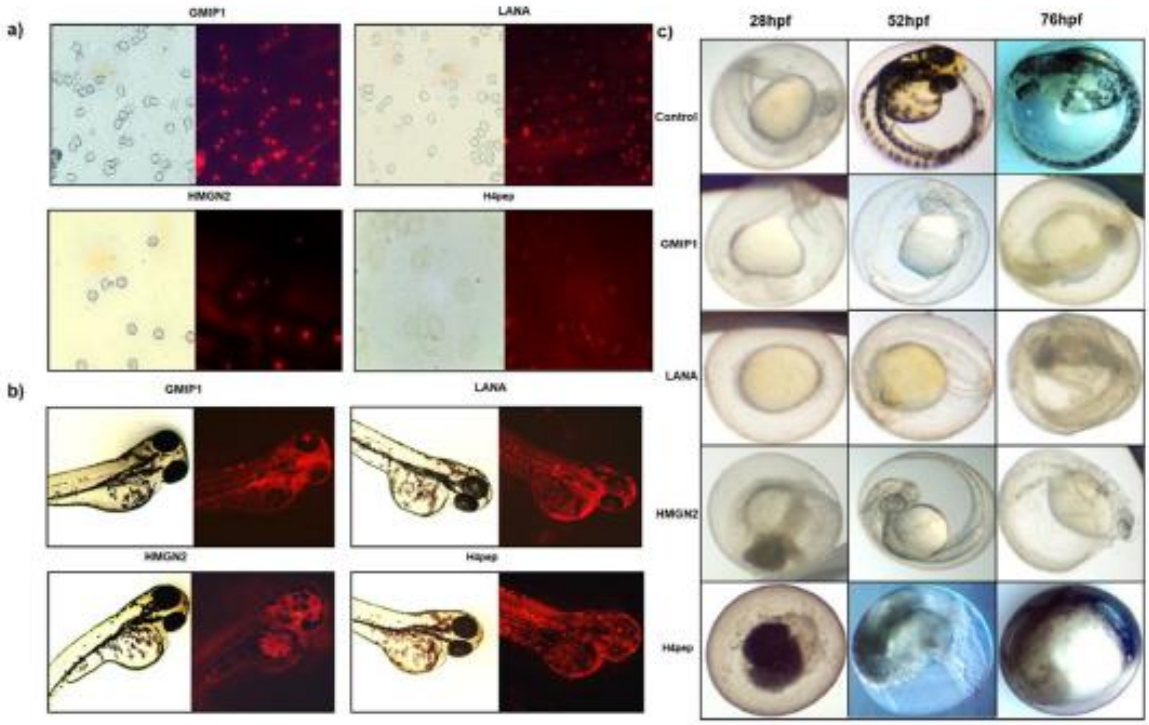

Fig. 5. NBPeps action *in vivo* a) Injection of fluorescent NBPeps accumulate in the nucleus erythrocytes of adult zebrafish, b) fluorescent NBPeps incubated for 3 h distributes heterogeneously over zebrafish larvae. At right panel, visualization using 520 nm laser. c) Fish Embryo Toxicity (FET) with NBPeps. Zebrafish embryos at 4, 28 and 52 h post fertilization (hpf) were incubated with NBPeps or vehicle for 24 h. Images are representative of three separate experiments.

required to bind in shallow crevices that mediate protein-protein interactions. Recently, binuclear ruthenium compounds was shown to be able to target the nucleosome surface and induce chromatin condensation [32].

Herein, we rationalized that NBPeps would be important candidates for occupying the nucleosome surface and to direct control the chromatin status and phenotypic outcomes. For that, NBPeps derived from binding motifs of structure-characterized nucleosome binding proteins, LANA, H4 tail, HMGN2 and the newly generated GMIP1, were tested to verify their ability in impacting the nucleosome stability, chromatin status, cell viability and fish embryo development. The four NBPeps bound to the nucleosome and induce precipitation at uM order, except H4pep that act at 500 nM.

We first searched for a novel NBPep that could bind to and present specificity for the nucleosome, with high dependence on nucleosomal DNA for the nucleosome binding. This characteristic could potentially open an avenue for the design of NBPeps with high specificity for target genes. However, the biochemical data shows that GMIP1 has low nucleosome binding affinity. It is important to emphasize that all experiments performed were done with the Widom 601 DNA sequence, which is an artificial sequence with high specificity to the octamer.

In general, the NBPeps displaced at least 10 % of the water associated with the acidic patch. Interestingly, thermal shift assays suggest that GMIP1 and HMGN2pep, which also bind to the nucleosomal DNA, primarily disrupt H2A:H2B dimer. This is partially in agreement with the molecular dynamic experiments, in which the HMGN2pep increased histones atomic fluctuations and reduced DNA fluctuation changes at the nucleosome entry-side DNA region. LANA firstly disturb H3:H4 tetramer and H4pep induced H2A:H2B dimer stabilization but did not affect H3:H4. Together with the notion that DNA is needed for GMIP1 binding led us to consider the hypothesis that NBPep with a DNA nucleosomal binding site might be by more effective for modifying the common target on the nucleosome surface. Chromatin compaction was affected by the H4pep but not by GMIP1, supporting the idea that GMIP1 presents low affinity binding to the nucleosome. Cell-based assays demonstrated that all four NBPeps were able to penetrate the cell, with GMIP1 having the best cell uptake. The protein-derived NBPeps, but not the rationally designed GMIP1, significantly affected the viability of tumoral cells. However, NBPeps had weaker effects on the cellular viability of non-tumoral cell lineage, except for H4pep that still showed strong negative effects on cell viability.

Experiments performed in zebrafish showed that NBPeps have a differential interference in the embryo mortality, development, pigmentation and hatching. Labeled NBPeps penetrated different tissues of fish larvae and localized in the nuclei. These results are in agreement to the cell-based assays showing the NBPeps penetrate the cell. It is worthy of attention that GMIP1 did not induce fish embryos mortality, contrarily to H4pep. These data corroborate the results from cell-based assays MTT using non-tumoral ccd 10595k cells. Interestingly, all NBPeps induced earlier hatching, but GMIP1 and LANA induced development embryo delay.

Taken together the *in silico*, biochemical an *in vivo* data, however, it is too early to provide a straight correlation between NBPeps binding sites and the phenotypic outcome. Considering that three out of the four designed peptides are non-specific nucleosomal interactors, a question that is still to be explored is whether the NBPeps may engage different signaling events, besides the nucleosome binding, for example affecting function of HDAC.

Indeed, acetylated H4 N-terminal tail was explored as a molecular tool to establish and maintain the active state of p53 target genes *via* interaction with histone deacetylase 1 (HDAC1). It was suggested that it could be used as a novel strategy for anticancer therapy [33]. Herein, we further advance the idea that H4pep could affect tumoral cells, but through the direct binding to the nucleosome surface, since H4pep was

the unique high specific NBPep, causing dramatic effect *in vivo*. Its effect emphasized the physiological role of H4 tail on chromatin condensation and transcriptional outcome.

In conclusion, we observed that NBPeps with distinct nucleosome binding sites perturb the nucleosome structure in multiple ways. Despite sharing an apparently similar target, NBPeps showed different roles in cell physiology, which is probably due to the non-specificity in targeting the nucleosome surface. However, new biophysical experiments in cell-based context should be performed to be able to straight correlate the *in silico* predictions with the *in vivo* findings.

Nevertheless, considering that DNA intercalaters or damaging agents still have great importance in clinical oncology, the fact that NBPeps do not present specific targeting would not preclude their use as therapeutic agents. Indeed, we believe that NBPeps open novel opportunities to design hybrid molecules with higher specificity to regulate a plethora of cellular disorders.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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21. APPENDIX B

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Review

Fat nucleosome: Role of lipids on chromatin

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ABSTRACT

Structural changes in chromatin regulate gene expression and define phenotypic outcomes. Molecules that bind to the nucleosome, the complex of DNA and histone proteins, are key modulators of chromatin structure. Most recently, the formation of condensed chromatin regions based on phase-separation in the cell, a basic physical mechanism, was proposed. Increased understanding of the mechanisms of interaction between chromatin and lipids suggest that small lipid molecules, such as cholesterol and short-chain fatty acids, can regulate important nuclear functions. New biophysical data has suggested that cholesterol interacts with nucleosome through multiple binding sites and affects chromatin structure in vitro. Regardless of the mechanism of how lipids bind to chromatin, there is currently little awareness that lipids may be stored in chromatin and influence its state. Focusing on lipids that bind to nuclear receptors, clinically relevant transcription factors, we discuss the potential interactions of the nucleosome with steroid hormones, bile acids and fatty acids, which suggest that other lipid chemotypes may also impact chromatin structure through binding to common sites on the nucleosome. Herein, we review the main impacts of lipids on the nuclear environment, emphasizing its role on chromatin architecture. We postulate that lipids that bind to nucleosomes and affect chromatin states are likely to be worth investigating as tools to modify disease phenotypes at a molecular level.

1. Chromatin and regulators

Genetic information is structurally organized within chromatin, which dynamically regulates how, when and where gene expression occurs in the cell. Besides the regulation of gene expression, chromatin is also responsible for regulating genome maintenance [1]. Highly basic proteins and DNA combine to form the nucleosome core particle (NCP) thus generating the fundamental repetitive unit of chromatin. The NCP represents the first level of DNA compaction, followed by a cooperative nucleosome interaction to form higher-order chromatin structure. The current hierarchical model is that 11-nm fibers composed of DNA-nucleosome polymers may fold into 30-nm fibers that further fold into 120-nm, 300-nm to 700-nm chromatids and, ultimately, into mitotic chromosomes. However, it was recently demonstrated, using a new DNA-labeling method that chromatin is not as well organized as previously thought; it comprises a flexible and disordered 5–24-nm granular chain that is packed together, thus challenging the long-standing textbook model of higher-order chromatin structure [2].

In an open state, chromatin is more susceptible to loading with the proteins that trigger transcription initiation, DNA repair and processes that regulate genome stability. In contrast, the close state of chromatin, heterochromatin, may provide steric exclusion of transcription factors and regulatory proteins such as nuclear receptors and RNA polymerases from target DNA, leading to gene silencing [3]. Indeed, the relaxed and the condensed chromatin structures provide the cell with a strategic plan to sharply regulate the access of proteins to DNA. Nevertheless, this is a simplistic picture, given that chromatin structure dynamics alone is not enough to produce predictive patterns of gene expression and phenotypic outcomes [4]. Moreover, the properties of the nuclear molecules that affect chromatin structure and fundamental processes, such as genome stability and transcription remain to be fully-characterized.

Chromatin dynamics are determined by a plethora of Nucleosome Binding Proteins, NBPs, several of which have already been characterized at the atomic level [5]. In addition to NBPs, the nuclear environment also comprises many small molecules with different chemical natures that can directly interact with nucleosomes, such as $Mg2+$ and lipids [6,7]. Thus, the molecular composition of the nuclear environment may have considerable impact on chromatin architecture, indeed, a phase diagram of the NCP published over a decade ago suggests that slight changes in ionic conditions may have significant impacts on nucleosome:nucleosome interactions [8].

More recently, an elegant hypothesis to explain the formation of heterochromatin regions was proposed by two different groups based

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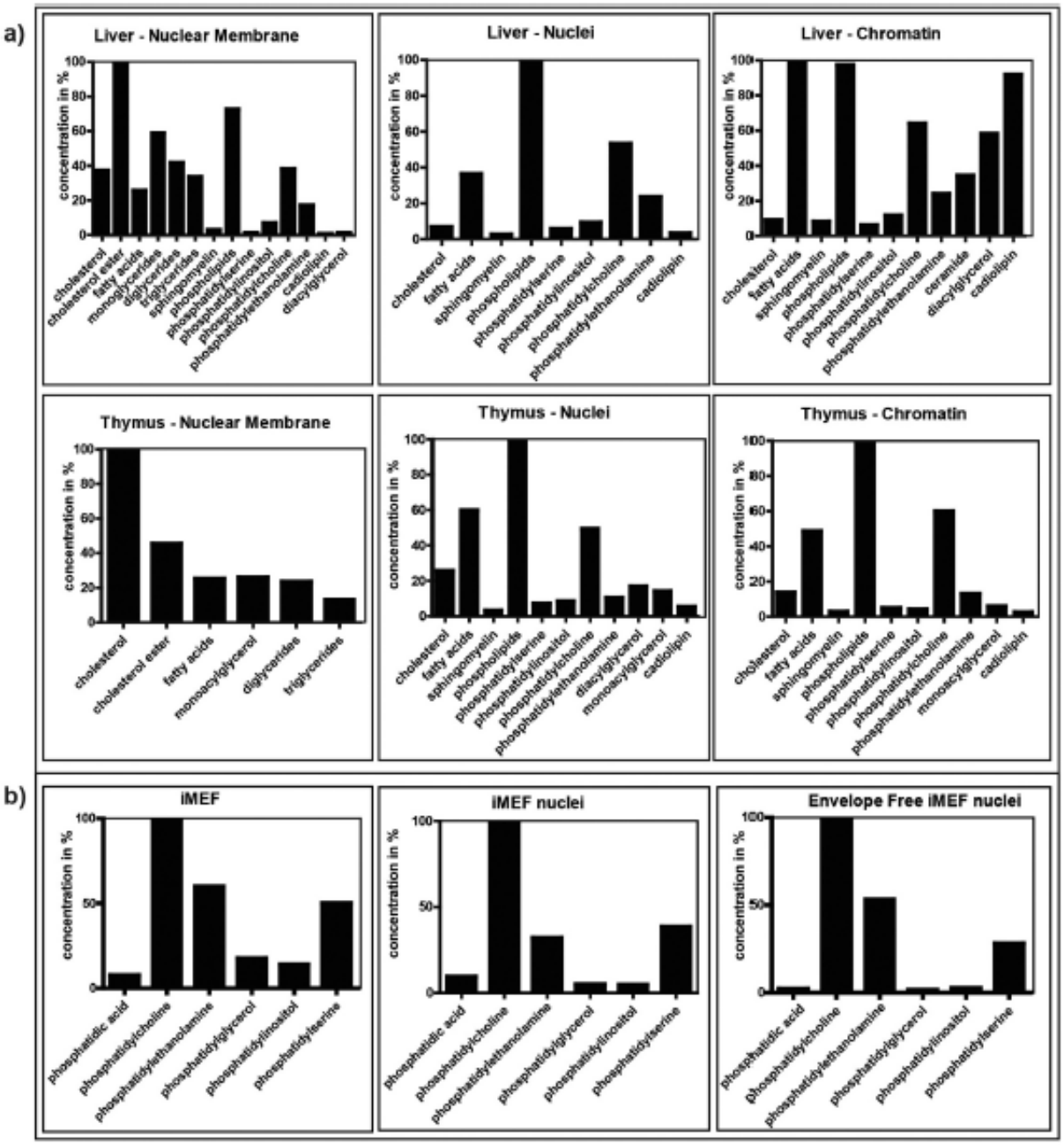

Fig. 1. Lipidic profile of nuclear compartments in liver, thymus and embryonic cells. A- The concentration of lipid is expressed in relation to protein:tissue, normalized in percentage related to the highest lipid concentration, from liver and thymus cells. Nucleus includes data from whole nuclear environment, including chromatin and nuclear membrane. References: Liver chromatin: [18,19,21] Liver nucleus: [18,19] Thymus chromatin: [20,22–24,27,28] Thymus nucleus: [22–25,27,28] Liver nuclear membrane: [17,19,20] Thymus nuclear membrane [20]. B- The concentration of phospholipids is expressed in percentage related to phosphatidylcholine from immortalized embryonic fibroblasts [26].

on phase-separation in the cell, a basic physical mechanism [9,10]. It was shown that a linker histone HP1, which is known as a key factor for inducing chromatin compaction, has the ability to form phase-separated droplets. Native, unmodified HP1 is soluble but phosphorylation of its N-terminal domain and binding to DNA induce the formation of phase-separated droplets. This process would potentially be responsible for the formation of compartments in the cell that do not possess a membrane or other physical barrier, and, in turn mediate gene silence induced by HP1 heterochromatin formation. This mechanism of segregation is analogous to oil de-mixing from water. While it seems clear that HP1 droplets are able to physically sequester chromatin, inducing compaction, the precise nature of the physico-chemical environment of

the phase-separated heterochromatin remains obscure.

Nuclear lipid droplets were described in 2012 as potential subnuclear domains. These droplets which as well as lipid also contain proteins, and these could provide a repository for the storage of lipids in the nucleus [11].

Increased understanding of the mechanisms of interaction between chromatin and lipids suggest that small lipid molecules, such as cholesterol and short-chain fatty acids, can regulate important nuclear functions. The role of lipids on the nuclear environment is prominent in protein:lipids complexes, such as Nuclear Receptors and their ligands. However, there is no clear perception of whether lipids may impact the nucleosome and chromatin structure, regulating gene expression and genome maintenance. Herein, we review the concentration of several lipids in the nuclear environment of different tissues, highlighting the lipids bound to chromatin. We also briefly present some ligands of nuclear receptor, discussing how they could bind to the nucleosome and chromatin. Understanding the role of lipids on the nuclear environment may be critical to comprehend how chromatin states are determined and how these states may subsequently affect phenotypic outcomes.

2. Lipids in the nuclear environment: lipids and chromatin

Lipid droplets are recognized as repositories for the storage of lipids for use on demand and they also participate in several physiological processes. Although well characterized in the cytoplasm, the existence of nuclear lipid droplets (nLD) has been recently described, functioning as a modulator of lipid metabolism in the nucleus. Lipid droplets interact with numerous proteins and specifically participate in the regulation of nuclear events [12]. Indeed, histones interact with lipid droplets during oogenesis in Drosophila embryos, which causes histone sequestration outside chromatin during embryogenesis [13].

In 2013 [11], nuclei from rat hepatocyte were separated from cytosol using sucrose gradient ultracentrifugation, neutral lipids contained in nLDs were stained and visualized with confocal microscopy. These nLDs were organized around a hydrophobic core of triacylglycerol and cholesteryl esters with oleic acid, surrounded by further cholesteryl esters, polar lipids and proteins, but the protein composition of nLDs has not been fully characterized.

Cascianelli et al. [14] have shown that nuclear lipid microdomains, containing sphingomyelin, cholesterol and proteins in the inner nuclear membrane, may function as scaffolds for RNA synthesis. The same group hypothesized that nuclear lipid microdomains could act as sites for chromatin anchorage, which would have important functions during the cell cycle. Moreover, it was shown that the tethering of chromatin to the nuclear periphery plays a crucial function in the chromatin flow by changing the nuclear shape [15].

Seminal studies in rabbits and rats have shown the presence of different lipids, cholesterol, fatty acids and phospholipids in the nuclear environment and their concentrations have been determined. It has been proposed that the ratio of cholesterol to phospholipids would have a great structural impact in the nucleus, rather than just a metabolic function [16]. Data for the concentration range of lipids in the different compartments in the nuclear environment is available for different tissues, herein we focus on the liver and thymus cells that literature vastly covered, and these are summarized in Fig. 1 [17–26]. In addition, we also analysed data from murine embryonic fibroblast, which was one of the most thorough examinations, using electrospray ionization mass spectrometry (ESI-MS), to determine the intra-nuclear lipid content of cells (Fig. 1b).

The precise determination of lipid concentration in the cell nucleus is clearly challenging, with several studies having found different values for the same lipid in the same conditions [22,27–29]. Herein, the analyses were made based on the relation of protein:lipid or protein:tissue showed in the articles. However, in this way it was difficult to make a clear qualitative view of the distribution of the lipids in the cell. Therefore, to have a clearer picture of the lipids distribution, the y-axis, which gives the concentration, was converted to percentage, considering 100% as the most prevalent lipid in each cell compartment (Fig. 1a) or in percentage related to phosphatidylcholine (Fig. 1b). This allowed us to compare the amount of different lipids in the same nuclear environment. In thymus and liver cells, predominately phospholipids are associated with chromatin. Indeed, phospholipids appeared to be the most abundant lipid, regardless of cell type and nuclear compartment (Fig. 1b) (detailed phospholipids characterization is found in [26]). Interestingly, cardiolipin, which was found to be almost exclusively in the inner mitochondrial membrane, is the third most abundant lipid associated with liver chromatin, in contrast to thymus that has very low cardiolipin concentration. Taking all together, it is noticeable that the distribution of lipids is cell type dependent.

The relevance of different lipid concentrations for intracellular signal during the cell cycle has been debated for a long time, suggesting a role for these molecules as structural parts of the chromatin [30,31].

Importantly, in vitro studies demonstrated that neutral lipids can bind specifically to the double helix DNA, but not triplex DNA. Spectroscopic and surface plasmon resonance studies indicate that linoleic acid is able to tightly bind to the minor groove of the double helix DNA [32]. If these findings are representative of the physiological reality in the nuclear environment, binding of lipids might have a profound impact on modulation of gene expression.

Following these findings, several more studies have attempted to elucidate the potential function of many lipids in the nucleus [29]. Mainly, gene expression was shown to be regulated by different lipids such as gamma linoleic acid and phosphatidylserine. Furthermore, a significant role for lipids on DNA polymerase modulation and DNA structure was demonstrated, and this would directly affect the transcriptional process.

2.1. Lipids and gene expression: steroids hormones, bile acid and fatty acids

It is well established that lipids regulate gene expression through transcription factors, such as nuclear receptors (NRs). NR ligands include lipids, such as cholesterol metabolites and fatty acids [33]. Moreover, phospholipids, one of the most abundant lipid molecules in the nucleus, may directly regulate NRs activity, evidencing an important action of these non-cholesterol-based molecules in modulating gene expression (reviewed in [34]).

Herein, we briefly point out some of the members of NR superfamily, which have lipids as ligands, to emphasize the biological and clinical relevance of its lipids in the nuclear environment, and provide references for more detailed information.

This superfamily of receptors is highly clinically relevant. The classical view of NR mechanism of transcription regulation is that NRs are activated by their ligands, bind to the promoter/enhancer region of target genes and recruit co-regulators to modulate both transcription activation and transcription repression [35]. We focus on three nuclear receptors that have lipids as ligands, peroxisome proliferator-activated receptors (PPARs), Liver X Receptor (LXR), Farnesoid X Receptor (FXR) and Estrogen Receptor (ER).

PPARs are involved in adipogenesis and lipid and carbohydrate metabolism, with high clinical relevance as insulin sensitizers [36]. PPARα is a key regulator of hepatic lipid metabolism trough the modulation of several genes related to mitochondrial and peroxisomal fatty acid β-oxidation. PPARα is also involved in the inflammatory pathway and glucose metabolism. PPARδ function is crucial for the glucose metabolism in skeletal, heart and pancreatic β-cells [37]. PPARγ is important for cellular differentiation, lipogenesis and insulin sensitivity in adipose tissue [38]. In 2008, structures of PPARγ bound to oxidized fatty acids were solved, suggesting that these fatty acids are natural ligands for this receptor. Interestingly, it was shown that PPARγ can bind two fatty acids at the same time and couple covalently with conjugated oxo fatty acids, which would be particularly effective activators of PPARγ [39]. Importantly, a component of Low Density

Lipoprotein (LDL) particles, Oxidized phosphatidylcholine (oxPC), is also able to bind and modulate PPARα [40] and PPARγ [41]. Although some phospholipids are found in the nuclear environment at lower levels, its importance on NRs activity, such as 1-Palmitoyl 2-Oleyl PC (POPC) on PPARα, has been demonstrated [42].

Another nuclear receptor, LXR (Liver X Receptor), which is highly expressed in the liver and adipose tissue, is also target for lipids such as oxysterols and cholesterol derivatives. LXR is able to target genes involved in cholesterol metabolism and is considered a sensor for intracellular cholesterol levels [43,44]. LXR activation may regulate target genes that lead to decrease cholesterol and increase HDL levels in the plasma [45,46].

FXR (Farnesoid X Receptor) has bile acids as ligands. This nuclear receptor may activate the VLDL receptor gene and other genes involved in the metabolism of lipoproteins [47]. It is expressed in the liver, kidney and intestines. FXR was described as a sensor for regulation of bile acids in the enterohepatic circulation [48,49].

Besides those NRs, the ER (Estrogen Receptor) is a key transcription factor that is regulated by steroid hormones, which are also classified as lipids. Selective ER ligands are highly promising agents for the treatment of the majority of breast cancers, as well as for cardiovascular, inflammatory, and neurodegenerative diseases. The ER is critical for the regulation of genes engaged in cell growth and endocrine response [33,50].

Although some of NRs ligands play a crucial role as second messengers, it is accepted that this extracellular molecules may also act as classic first messengers on NRs. From these examples amongst Nuclear Receptors briefly discussed here, it is clear that many lipidic ligands can regulate cell functioning, especially gene expression, via transcription factors present in the nucleus. Considering that these ligands may permeate the nuclear environment, it is plausible to believe that they could also bind the lipophilic regions of chromatin affecting its structure and, ultimately, gene expression. Moreover, it is probable that chromatin may store lipids that can act as NR ligands, which would then be available according to the NR's requirement.

3. Complexes of lipids with the nucleosome: cholesterol, steroids hormones, bile acid and fatty acids

In 1975, chromatin fibers purified from rats treated with labeled cholesterol revealed the association of this macromolecule with cholesterol [51]. Later, it was suggested that cholesterol binds to chromatin through one or more proteins rather than to nucleic acid [52].

Recently, some biophysical effects of cholesterol on chromatin were demonstrated [7], suggesting that cholesterol binds directly to chromatin and facilitates in vitro formation of mononucleosomes and chromatin fibers. Importantly, cholesterol induced the compaction of open chromatin fibers (Fig. 2). Molecular Dynamics experiments suggested six binding sites for cholesterol on the nucleosome. For all of these sites, the estimated binding free-energies indicated that cholesterol interacts with the nucleosome with low affinity. By binding at these locations cholesterol resided near amino acids from key nucleosome regions, such as H4 tail, the acidic patch (a negatively charged region on the nucleosome surface formed by six H2A and two H2B residues) and the docking domain in between the histone clusters H3-H4 and H2A-H2B (Fig. 3). Further computational comparative analysis in presence and absence of cholesterol led to the hypothesis that cholesterol effects on chromatin could be due to (i) dewetting of nucleosome surface that might enhance interactions between key structural hydrophobic amino acids and (ii) an additional mechanism involving specific binding locations of the H4 tail, known for internucleosomal interactions.

Moreover, as previously discussed, other small lipid molecules are present in the nucleus, and also are likely to interfere with chromatin structure [53]. Therefore, presumably other lipids types could affect chromatin structure through direct interaction with the nucleosome.

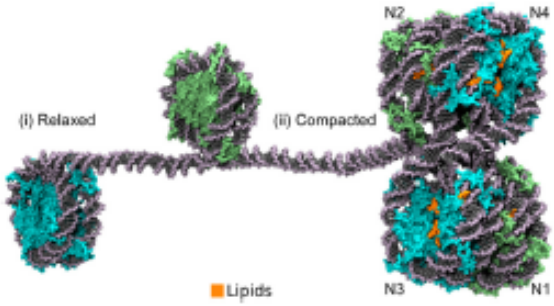

Fig. 2. Pictorial model for the potential role of cholesterol in modulating chromatin compaction. Two states of chromatin are shown, (i) relaxed fiber (left), in which the nucleosomes are more distant from each other; (ii) and compacted fiber (right), where the presence of cholesterol (orange) results in heterochromatin formation. The histone octamer are represented in blue and green to distinguish the nucleosomes. The tetranucleosome is numbered from N1 to N4 in the compacted state. The compacted fiber model was generated from the tetranucleosome structure revealed by Schalch and colleagues [62]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Consistent with that notion, exploratory docking calculations between lipid-chemotypes, other than cholesterol, and the nucleosome suggested that cholesterol and other lipids may bind at common sites on the nucleosome surface (unpublished data). Although preliminary, these calculations support that cholesterol binding sites can be promiscuous allowing binding of other lipids, including steroids hormones, bile acid, fatty acids and phospholipids. In the case of Nuclear Receptor ligands, they could act directly via chromatin, and not just on Nuclear Receptors. Furthermore, it may suggest that chromatin can function as a repository or depot, for these lipids, becoming available to NRs when they are needed.

Several other small-molecules have already been characterized as binding to chromatin and theoretical and molecular techniques have been very useful to show the interaction of ions and small peptides with chromatin. Fan and co-workers used Molecular Dynamics simulations of coarse-grained nucleosomes (simplified representation of chromatin compared to the classic atomistic models) to demonstrate that divalent ($Mg2+$) and trivalent ($CoHex3+$) ions resulted in aggregates of NCPs. For the trivalent ion, all 10 NCPs simulated could form aggregates, inducing phase-separation [54]. In addition, divalent metal ions bind to chromatin and may compete with histone tails to access the DNA groves, which could alter the chromatin states [55]. Taken all together, this variety of molecules that interact with chromatin reinforce the view that chromatin could represent a depot for many different small molecules, in addition to the lipids which are the focus of this review.

4. Clinical outcome of lipids and chromatin interaction

Lipids play essential roles in the cell and a wide range of diseases has been associated with lipid metabolism. Plasma cholesterol and triglyceride levels are used as predictors of metabolic disorders and vascular disease risk and have been crucial for physicians over the past century [56]. However, it is still unknown how lipids with multiple functions such as cholesterol that is essential for steroid hormone production, the structural integrity of cellular membranes, and cell growth, may affect chromatin structure and, ultimately, clinical outcomes.

What we know so far is that cholesterol and other lipids may have a direct impact on the chromatin architecture in vitro, triggering changes that may contribute the intra-nuclear organization of chromatin [7]. It is also clear that the understanding of changes in chromatin dynamics may uncover mechanisms of diseases and shed light on potential treatment alternatives [57]. Several histone post-translational modifications responsible for changes in the chromatin structure have been

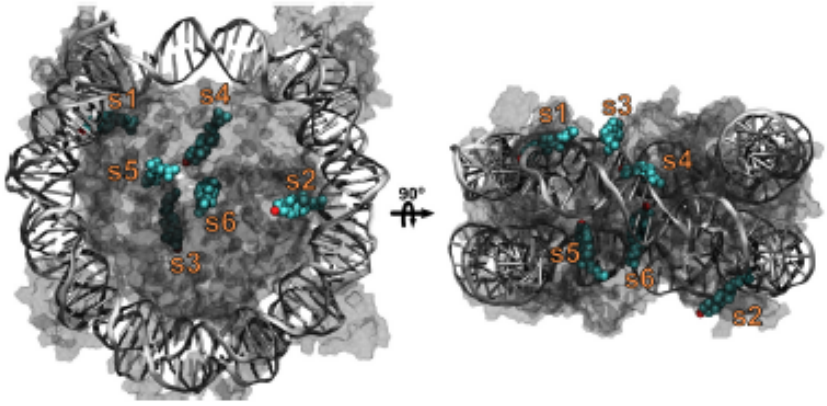

Fig. 3. Cholesterol binding on nucleosome surface. The nucleosome surface is shown (gray) with six cholesterol molecules (colored by atom type) bound at different regions (sites s1 to s6). Adapted from [7].

associated with cancer and other diseases, directly associating chromatin architectural changes with disease mechanisms. The epidrugs [58], an emergent class of drugs, target enzymes and other proteins that are able to control epigenetic changes and hence gene expression for disease treatment. Observing the great clinical importance of epidrugs, it is plausible to consider that any molecule that interacts with the nucleosome and affects chromatin dynamics would also be able to cause disease phenotypes. For the time being it is unclear if the actions of lipids on chromatin are potential therapeutical targets. The structural characterization, at atomic level, of the lipids binding mode to nucleosome could provide new insights in the development of hybrid molecules for modulating chromatin structure [59].

Moreover, detailed information about the nuclear environment under different physiological and pathological states is needed before this field can advance significantly toward clinical utility. It will be very important to precisely determine the variations in nuclear lipid concentrations and localization during the circadian rhythm, following the chromatin structural changes and nuclear localization and finally correlating them with transcriptional activity and phenotypic outcome. Understanding how lipids act on chromatin is a promising avenue for the development of new treatments.

5. Concluding remarks and future perspectives

The modern view of chromatin modulation by NBPs and post-translational modifications of histones must be updated. An avalanche of data concerning the manner of chromatin dynamics leads us to re-interpret the simplistic scenario, in which protein complexes dictate chromatin states.

The roles of several NBPs, key players in modulating chromatin structure, have been well characterized at atomic level. These structures revealed details of the interaction of NBPs with the nucleosome surface, highlighting the acidic patch as the principal protein-docking region. However, this region does not seem to be exclusive for NBPs. In silico experiments found multiple binding sites for cholesterol on the nucleosome, including the acidic patch.

Mounting evidences support that different lipids, such as cholesterol, fatty acids and phospholipids may interact directly with a number of nuclear protein structures to impact their function in a specific and concentration-dependent manner. Exemplars of nuclear protein are Nuclear Receptors superfamily, which was briefly discussed here. Differently, lipids appear here to interact with chromatin in a promiscuous non-specific way, through multiple low-affinity receptor sites. Binding energies as estimated from docking calculations support that lipid-chromatin interactions are low affinity, in the range of 10^{-5}mM (unpublished data). Although an entire new series of high-resolution experimental and theoretical studies, as done with cholesterol, will be needed to shed light on the molecular nature of lipid-chromatin interactions. However, these preliminary observations thus support that lipid-chemotypes, may bind at common sites on the nucleosome surface. Still consistent with that notion is the fascinating possibility that via non-specific low-affinity interactions, chromatin may work as a lipid depot for nuclear proteins.

The 4D Nucleome Network [60] was recently presented as a potential third phase of the human genome project, aiming to develop and use a wide array of technologies to characterize the structure and dynamics of the human and mouse genomes. The main idea is to integrate several disciplines, from biology to mathematics, to decipher how the chromatin is organized and function inside the nucleus in space and time. It seems a fantastic and ambitious project, which will render many new insights that can ultimately be useful to understand how the human genome is packed in the cell in a functional way.

However, several questions regarding the molecules that compose the nuclear environment, and may affect chromatin architecture, remain to be answered. Firstly, getting a clear map of the contents in the nuclear environment in different conditions would provide us with the opportunity to make solid correlations with many events in the cell, including chromatin states, gene expression and DNA stability processes. At the same time, better characterization of substances, such as lipids, ions and a plethora of proteins that drive phase-separation properties on the chromatin structure is necessary. Independently whether the lipids retain or not a direct action on chromatin, we should at least consider that under a certain concentration a hydrophobic molecule would be able to affect the manner that macromolecules, such as the nucleosome, will interact with each other and, consequently, modulating its state.

At the same line of research, it was shown that protein domains of low-complexity, which don't comprise a balanced proportion of all 20 amino acids, may polymerize into cross-β structure. This, in turn, can be the driving force for hydrogel and liquid-like droplet formation [61]. We could then question whether lipids could cooperate with protein-based hydrogels formation and achieve the same role as the phase separations in the cell, modulating the chromatin architecture.

Lately, by reasoning that any molecule that binds to the nucleosome and affects the chromatin state is able to tackle disease phenotypes at a molecular level, we anticipate that correlations of the outcome from the 4D nucleosome project with a map of nuclear molecules in certain space and time will advance rational drug design and development of new treatments for a wide range of diseases.

The rapid development of various technologies, from computational to structural biology, has occurred in the past decade to investigate the role of many substances on chromatin. Hopefully, the combination of

all of these areas will assist to overcome the arduous tasks of obtaining a clearer overview of cell function.

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21. APPENDIX C

REV BRAS REUMATOL. 2017;57(6):596–604

REVISTA BRASILEIRA DE REUMATOLOGIA

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Review article

Cyclophosphamide administration routine in autoimmune rheumatic diseases: a review☆

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ABSTRACT

Cyclophosphamide is an alkylating agent widely used for the treatment of malignant neoplasia and which can be used in the treatment of multiple rheumatic diseases. Medication administration errors may lead to its reduced efficacy or increased drug toxicity. Many errors occur in the administration of injectable drugs. The present study aimed at structuring a routine for cyclophosphamide use, as well as creating a document with pharmacotherapeutic guidelines for the patient. The routine is schematized in three phases: pre-chemotherapy, administration of cyclophosphamide, and post-chemotherapy, taking into account the drugs to be administered before and after cyclophosphamide in order to prevent adverse effects, including nausea and hemorrhagic cystitis. Adverse reactions can alter laboratory tests; thus, this routine included clinical management for changes in white blood cells, platelets, neutrophils, and sodium, including cyclophosphamide dose adjustment in the case of kidney disease. Cyclophosphamide is responsible for other rare – but serious – side effects, for instance, hepatotoxicity, severe hyponatremia and heart failure. Other adverse reactions include hair loss, amenorrhea and menopause. In this routine, we also entered guidelines to post-chemotherapy patients. The compatibility of injectable drugs with the vehicle used has been described, as well as stability and infusion times. The routine aimed at the rational use of cyclophosphamide, with prevention of adverse events and relapse episodes, factors that may burden the health care system.

☆ Study conducted at the Serviço de Reumatologia, Hospital Universitário, Universidade de Brasília, Brasília, DF, Brazil.

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Rotina de administração de ciclofosfamida em doenças autoimunes reumáticas: uma revisão

RESUMO

A ciclofosfamida é um agente alquilante vastamente usado para o tratamento de neoplasias malignas e pode ser usado no tratamento de diversas doenças reumatológicas. O erro de administração de medicamentos pode levar à diminuição da eficácia ou ao aumento da toxicidade medicamentosa. Diversos erros ocorrem na administração de medicamentos injetáveis. O trabalho objetivou a estruturação de uma rotina do uso de ciclofosfamida, bem como a criação de um documento de orientações farmacoterapêuticas para o paciente. A rotina foi esquematizada em três fases, a pré-quimioterapia, a administração da ciclofosfamida e a pós-quimioterapia, que levaram em consideração os medicamentos que devem ser administrados antes e depois da ciclofosfamida para prevenção aos efeitos adversos, incluindo náusea e cistite hemorrágica. As reações adversas podem alterar os exames laboratoriais e a rotina incluiu manejo clínico para alteração clínica dos leucócitos, das plaquetas, dos neutrófilos e do sódio incluindo o ajuste de dose de ciclofosfamida em caso de insuficiência renal. A ciclofosfamida é responsável por outras reações adversas raras, mas sérias, como hepatotoxicidade, hiponatremia severa e falência cardíaca. Outras reações adversas incluem perda de cabelo, amenorreia e menopausa. A rotina foi composta também por orientações ao paciente pós-quimioterapia. A compatibilidade dos medicamentos injetáveis com o veículo foi descrita, bem como o tempo de estabilidade e o tempo de infusão. A rotina visou ao uso racional da ciclofosfamida e prevenir os efeitos adversos e os episódios de recidiva, os quais podem onerar o sistema de saúde.

Palavras-chave:
Ciclofosfamida
Antieméticos
Quimioterapia
Cistite

Introduction

Cyclophosphamide (CPM) is an alkylating agent widely used for the treatment of malignancies such as breast cancer,[1] multiple myeloma,[2] renal diseases including nephrotic syndrome refractory to corticosteroid and focal segmental glomerulonephritis, and this drug can be used in the treatment of multiple rheumatic diseases,[3–5] including cicatricial pemphigoid (also called pemphigoid mucous membrane),[4] rheumatoid arthritis,[5] juvenile dermatomyositis,[6] systemic sclerosis,[7,8] interstitial lung disease,[7] lupus vasculopathy,[9] systemic vasculitis, and refractory treatment of lupus-associated thrombocytopenic purpura.[10] In addition to other indications of cyclophosphamide, the treatment of neuromyelitis optica can also be included.[11]

In children, cyclophosphamide may be used in the treatment of nephrotic syndrome and systemic lupus erythematosus.[12,13]

Cyclophosphamide can be administered by oral or intravenous route.[14] The intravenous administration is more frequent in the field of rheumatology, taking into account studies showing an efficacy similar to that of oral treatment, but with less toxicity, for example, a decrease in premature ovarian failure, less severe infection, and lower overall exposure of the urinary tract to acrolein, a toxic metabolite of cyclophosphamide.[15] Cyclophosphamide is orally administered QD (24–24 h), while the intravenous route is administered in pulses, and the dose is adjusted according to hematologic and renal toxicities.[16]

The administration of cyclophosphamide in pulses may follow a weekly or monthly basis, in combination with a corticosteroid and other chemotherapeutic agents, provided that the attending physician takes into account the minimum blood count (NADIR) for the administration of cyclophosphamide.[16–18] Cyclophosphamide may cause some adverse events, and when these effects are related to the drug, are classified as an adverse drug reaction.[19] The adverse drug reaction can be conceptualized as an unintended and harmful reaction into the body, occurring in those routinely used doses in humans for prophylaxis, diagnosis, disease therapy, or for changes of physiological functions.[19]

A reaction that occurs in a small percentage of the population, but that, if not avoided, may cause irreversible damage to the patient, such as death, congenital abnormalities, birth defects or conditions that require permanent hospitalization, is classified as "severe reaction".[19]

Some adverse reactions related to the administration of cyclophosphamide are bone marrow suppression, susceptibility to infections, sterility and amenorrhea,[18] as well as nephrotoxicity and cystitis,[18,19] and also cardiovascular complications, for instance, sinus bradycardia, pericarditis, myocarditis and heart failure.[20] Children and adolescents treated with high doses of cyclophosphamide are more likely to develop dental disorders and a decreased salivary flow. Cyclophosphamide is also teratogenic.[21] A long term reaction of cyclophosphamide is malignacies.[18] One can observe an increase in the incidence of bladder cancer and esophageal and lung adenocarcionoma, which customarily occur after two years of treatment.[18]

In addition to adverse drug reactions of cyclophosphamide, it is critical that the physician adopt all precautionary measures, because these adverse reactions may be more important in cases where cyclophosphamide is administered intravenously, given that, with this route of administration, the drug is not absorbed, the onset of its action is faster and, because the drug does not undergo first-pass metabolism, the bioavailability, i.e. the bioavailable concentration to exert a pharmacological action, becomes proportionally higher versus oral administration of cyclophosphamide.[22,23] Another important aspect to consider concerns the errors associated with the administration of injectable drugs.[24]

It is known that the use of injections is often associated with medication errors classified as serious events.[22] Intravenous-route administration errors represent 21.1% of all errors, with possible risk of errors of contamination, administration rate, and dilution.[25] Such parenteral drug administration errors, especially those by intravenous route, can cause an adverse drug reaction.[19]

In addition to health-related problems, it is required that the costs resulting from drug administration errors are taken into account. In Brazil, it is estimated that the annual cost with the use of chemotherapeutic drugs exceed 1.1 billion reais, and this value can increase in cases of injectable drug administration errors.[24]

Thus, one must bear in mind that these costs may be magnified by the occurrence of adverse events.[26] An adverse event is any untoward medical occurrence that affects the patient being medicated, but without a direct causal relationship with his/her treatment.[19]

Thus, it is important to standardize the administration of parenteral drugs and provide their rational use, which can be defined as the patient's need to receive the appropriate drug in the correct dosage for an adequate length of time and with the lowest cost.[27]

Taking into account the rational use of drugs, it is important to standardize rules for the parenteral administration of cyclophosphamide. The objectives of this study are proposing a cyclophosphamide administration routine in Rheumatology units, and the creation of a document containing pharmacotherapeutic guidelines for the patient, in order to maximize the efficiency of treatment based on a literature review.

Methods

Cyclophosphamide routine

An infusion routine for cyclophosphamide was developed in order to increase the bioavailability (effectiveness) of this drug while minimizing adverse reactions, thus rendering the treatment more tolerable for the patient. The protocol was divided into stages to facilitate the comprehension of the health staff and the replication of the cyclophosphamide dosing schedule. The drugs of the first (pre-ChT) and third (post-ChT) steps correspond to the administration of pharmaceuticals in order to prevent the main adverse reactions resulting from the administration of cyclophosphamide (second phase), including nausea, vomiting, and hemorrhagic cystitis. The clinical management of adverse reactions that change laboratory tests was described, as well as the clinical management of cyclophosphamide in renal failure. The rare, but serious, adverse reactions were also highlighted.

The most common adverse reactions were emphasized so that the attending physician could provide guidance with respect to the main care that the patient should take in his/her home, after administration of cyclophosphamide. These precautions are essential to prevent or minimize adverse effects and increase treatment adherence.

In addition, the compatibility of these injectable drugs with the vehicle, stability time, and infusion time was ascertained. The sequence of administration of these drugs has been prepared in order to increase the efficacy of cyclophosphamide and diminish the onset of adverse reactions to the drugs administered.

Results

The routine administration of cyclophosphamide was developed in three phases: pre-ChT, cyclophosphamide administration, and post-ChT. With a view to the prevention of hemorrhagic cystitis, intravenous hydration with a blood volume expander (a crystalloid: 0.9% saline) was standardized.[28] In addition, the drug administration sequence, the amount of diluent, the need (or otherwise) for dilution, infusion and administration times, and laboratory tests which should be monitored before and after the infusion of cyclophosphamide were indicated, as well as the guidelines to the patient.

Fig. 1 [3,5,20,29–42], lists the routine data, and Fig. 2 [44–50] describes the guidelines for the patient. Severe and uncommon adverse reactions which should be monitored are hepatotoxicity,[3] hyponatremia[45] (in this case, serum sodium level = 135 mmol/L [sodium is an univalent element; thus, 1 mmol/L = 1 mEq/L)],[45] and cardiovascular failure due to cyclophosphamide cardiotoxicity.[36]

Other adverse reactions from cyclophosphamide are amenorrhea,[18] early menopause,[46] and hair loss.[3]

Discussion

Drug administration errors can decrease the effectiveness of the pharmacological treatment and increase both the occurrence of adverse reactions and the financial costs of treatment.[24] A retrospective study conducted in hospitals in Spain between 2008 and 2010 evaluated the incidence of cost and adverse event. 245,320 episodes were identified, with an overall cost of 1,308,791.97 euros. Approximately 6.8% of patients experienced adverse events, representing a rise of 16.2% in costs. Six of the ten adverse events that burdened more significantly the hospital system occurred in the operating room, corresponding to an increase of expenses around 6.7% to the health system.[26]

A prospective study conducted between August and November 1999 and between January and May 2000 notified 1800 errors in 1663 patients. The number of notifications resulting from drug-related problems (including those probable and possible ones) was 215 (11.9%). Of these notifications, 108 (50.2%) were filled up due to adverse reactions, 100 (46.5%) came out of therapeutic failure related to dosing, and seven

Cyclophosphamide administration routine in autoimmune rheumatic diseases

Pre-chemotherapy administration scheme

Hydration with saline (S)
In the case of congestive heart failure, hypertension, acute/chronic kidney disease, use glucose solution. The infusion time must be 3h.

Specified dose: 1000 mL/S 0.9%[a] ☐	Dose: _____
Specified infusion: 1 hour/S 0.9%[a] ☐	Infusion time: _____
Specified time: 1 hour before cyclophosphamide ☐	Administration time: _____

Dexamethasone[b,c]

Specified dose: 20 mg ☐	Dose: _____
Dilution: 20 ml of S 0.9%[d] ☐	Dilution used: _____
Infusion: 10 minutes ☐	Infusion time: _____
Time: 1/2 hour prior to cyclophosphamide ☐	Administration time: _____

Mesna
The use of mesna is controversial; it may be replaced by a suitable hydration.[e]

Specified dose: 20% of the dose of cyclophosphamide[e] ☐	Dose: _____
Dilution: 20 ml of S[d] ☐	Dilution used: _____
Infusion: 15-30 minutes[e] ☐	Infusion time: _____
Time: 15 minutes prior to cyclophosphamide[e] ☐	Administration time: _____

The administration of cyclophosphamide is carried out immediately after pre-QT phase, with a dose between 0.5 and 1 g/m² body surface,[f,g] diluted into 100-200 ml saline,[h] with an infusion time of 60-120 minutes.[h]

Dosage scheme after chemotherapy

Furosemide[i]

Specified dose: 20 mg ☐	Dose: _____
Specified infusion: 1-2 minute bolus ☐	Infusion time: _____
Specified time: immediately after cyclophosphamide ☐	Administration time: _____

Mesna
The use of mesna is controversial; it may be replaced by a suitable hydration.[e]

Specified dose: 20% of the dose of cyclophosphamide (IV) or 40% of the PO dose[e] ☐	Dose: _____
Dilution: 20 mL S in the case of IV[d] ☐	Dilution used: _____
Infusion: 15-30 minutes ☐	Infusion time: _____
Time: 4 and 8 hours for IV use, or 2 and 6 hours for PO[e] ☐	Administration time: _____

Ondansetron[j]

Specified dose: 8 to 16 mg (VO) ☐	Dose: _____
Time: 6 and 14h after cyclophosphamide ☐	Administration time: _____

Suggestion to interrupt cyclophosphamide treatment, according to hematological parameters

Platelets <100 000/mm³ [k]
Absolute neutrophil count <1500 cells/µL [l]
White blood cell count <3500/mm³ [k]

Precautions and clinical management of cyclophosphamide with respect to genitourinary system

Kidney failure - *clearance* <10 mL/min: Reduce the dose by 25%; supplementation with 50% after dialysis.[m]

Urinalysis - obtain a urinalysis every 4 weeks after administration of cyclophosphamide because of the risk of hemorrhagic cystitis;[n] watch for signs of hematuria, proteinuria, and leukocyturia.[o]

Noninvasive and invasive laboratory tests must be monitored in patients receiving cyclophosphamide according to the severity of their condition.

Noninvasive methods - echocardiography, assessment of cardiac function (systolic ejection and diastolic function, diastolic peak velocity and atrial and valve function, electrocardiograms, electrocardiography, 24-hour Holter ECG).
Invasive methods - magnetic resonance imaging, myocardial biopsy.[p]

Serious and uncommon adverse reactions should be monitored:
Hepatotoxicity[q] and hyponatremia. Serum sodium concentration in this case is 135 mmol/L (sodium is an univalent element; thus, 1 mmol/L=1 mEq/L).[r] Cardiovascular failure due to cyclophosphamide cardiotoxicity.
Other adverse reactions from cyclophosphamide:
Amenorrhea,[s] premature menopause,[s] and hair loss.[q]

Fig. 1 – Cyclophosphamide administration routine in autoimmune rheumatic diseases.
Sources: [a]Shepherd et al.[29]; [b]Hawthorn; [c]Cunningham[30]; [d]Jordan et al.[31]; [d]Turner et al.[32]; [e]Trissel[33]; [f]Haubitz et al.[34]; [g]Mardegan et al.[35]; [h]Di Lisi et al.[36]; [i]Salido et al.[37]; [j]Calixto-Lima et al.[38]; [k]Lotan et al.[20]; [l]Zahn et al.[39]; [m]Milman[40]; [n]Mota et al.[5]; [o]McDermid and Lönnerdal[41]; [p]Centro de Oncologia Unimed Birigui[42]; [q]Subramaniam et al.[3]; [r]Mota et al.[43]

Water intake - the physician should instruct the patient to drink at least two liters of water a day unless in the presence of a kidney condition; thus, the amount of water to be consumed should be individualized. Imprensa electrolytes in the blood.[a]
Instructions for preventing mucositis - cyclophosphamide can cause mucositis; some precautions are advisable and these should be taken by the patient to minimize risks, including brushing adequately his/her teeth after a meal, using a soft brush and a non-abrasive toothpaste (for children), avoiding alcohol and cigarettes, avoiding too much salt, choosing soft foods, giving preference to food in the form of puddings, porridges, vitamins, gelatin, and caloric-, protein-rich meat, chicken or fish soup.[b,c]
Clinical management for symptoms of nausea - the patient should eat before getting hungry, with small, frequent (2-2 hours), slowly eaten meals because hunger can increase nausea; also, should avoid very spicy, fatty and sweet food and hot food and drinks. On the other hand, the patient should avoid drinking liquids during meals and should stay away from the kitchen during food preparation, eating in a ventilated and pleasant environment.[b]
Guidelines for patients with anemia - the patient should consume foods of animal origin such as chicken, fish and especially red meat, and should consume legumes and dark green vegetables such as kale, broccoli and spinach, pea beans and other grains, and combine vegetables with sources of vitamin C (orange, mandarin [tangerine], lemon, acerola). Milk, cheese, cottage cheese, yogurt and other dairy products should be avoided during or close to lunch or dinner time.[d,e]
Guidelines for disposal of urine and faeces - on the appointed day to take cyclophosphamide and on the next two days, when using the toilet the patient should flush three times with the cover closed. If someone else is responsible for the cleaning of the patient's excreta, this person should wear gloves and use disposable material. The cleaning procedure must be done in a outside-inside manner; and all content should be placed in two plastic bags, which will then be tightly closed. Cleaning should be completed with bleach.[f,g]
Guidelines for bathing - the patient must first wash his/her hands, then the face and head. Next, the stomach, back, arms and the catheter (if in use of one of these devices).

Fig. 2 – Guidelines for the patient taking cyclophosphamide.
Sources: [a]Instituto Estadual de Hematologia Arthur de Siqueira[44]; [b]Bruining et al.[45]; [c]González et al.[46]; [d]Otero López[47]; [e]Medeiros-Souza et al.[48]; [f]Mesna[49]; [g]Taketomo et al.[50].

(3.3%) were due to intoxication. According to the criteria (modified) of Schumock and Tornton, 68.4% of drug-related problems are considered as preventable.[47]

The cyclophosphamide dosing schedule took into account the most common adverse reactions, the administration's strategy in the case of kidney failure, and the sequence in the administration of pre-ChT drugs, cyclophosphamide, and post-ChT drugs; to this end, the dosage of all medications used, their dilution and infusion time also were included.

The main clinical strategies in the face of adverse reactions from cyclophosphamide use included the care of hemorrhagic cystitis, nausea, and vomiting.[18] The administration of these drugs corresponds to what is defined as a qualitative polypharmacy, wherein the administration of a medication is performed to correct the adverse reaction of another medication.[48]

Several clinical strategies have been proposed to avoid hemorrhagic cystitis, including increased hydration of the patient and the administration of mesna and furosemide.[28,29] In those cases in which the patient suffers of a severe kidney impairment, it is preferable to administer mesna in place of hydration, due to the patient's water restriction.[20,29]

For the prevention of hemorrhagic cystitis, mesna is administered in a dose equivalent to 60% of cyclophosphamide, divided into three doses – 20% 15 min before the administration of cyclophosphamide, 20% after cyclophosphamide, and 20% 4 or 8 h after cyclophosphamide.[28] Mesna reduces the deposit of acrolein (a metabolite of cyclophosphamide) in the bladder, thus preventing hemorrhagic cystitis and bladder cancer.[18]

In order to decrease the exposure of the urinary tract to acrolein, the patient should be well hydrated before, during and after the administration of cyclophosphamide. Thus, at the time of his/her admission, the patient should receive, through a venous access, 1 L of a blood volume expander (a crystalloid: saline 0.9%) for 1 h, 60 min before the administration of cyclophosphamide.[51]

Cyclophosphamide per se can be administered at any time. In turn, the infusion of mesna depends on the administration of cyclophosphamide.[28] Mesna may be administered by oral or parenteral (subcutaneous or intravenous) route.[28]

The oral administration of mesna has the advantage of a convenient dosage schedule; however, its use brings some disadvantages, for instance, a higher frequency of nausea and vomiting.[49,50] Another limiting factor of the use of this drug orally is the decreased bioavailability versus parenteral route, due to first-pass metabolism, in addition to the potential decrease in its absorption as a result of frequent episodes of vomiting caused by the treatment with cyclophosphamide.[31] Taking into account the concepts that guide the rational use of pharmaceuticals, including the reduction of treatment costs for both the patient and society, oral administration of

mesna would have also the advantage of a likely decrease in expenses, due the lower bed occupancy time and less workload of the nursing team.[51] Still in this context, another possible disadvantage is the patient's non-compliance; thus, one cannot be sure that the patient has taken, or otherwise, the last dose of mesna PO.[52]

The advantage of the use IV mesna is that there is no need of absorption, and the onset of action of this drug is faster, compared with oral administration.[22,23,53] The disadvantage of the parenteral route is the greater risk of contamination, administration errors, less dosing convenience, and increase of the patient's hospital stay.[23,53]

However, mesna administration with a view to the prevention of hemorrhagic cystitis in patients using cyclophosphamide in therapeutic doses for rheumatic diseases is controversial; thus, mesna may be replaced by a suitable hydration with 6 L of water per day, plus a diuretic drug, or the use of hydration with a volume of 3 L/m^2 per day.[54]

Furosemide is administered after cyclophosphamide infusion at a dose of 20 mg, in order to stimulate diuresis that, in synergism with mesna, decreases the exposure of urothelium to the action of acrolein.[55] The maximum concentration of furosemide is 10 mg/mL administered in bolus, achieving a therapeutic concentration of 10 mg/mL per minute.[55] The second dose of mesna (20% of cyclophosphamide dose) is administered in the interval between 15 and 30 min after the administration of cyclophosphamide.[28]

Nausea and vomiting are considered as common adverse reactions in chemotherapy, and this also occurs with cyclophosphamide[31] which, in turn, participates of many chemotherapeutic regimens. In this case, a routine has been proposed for the treatment of rheumatic diseases. Nausea caused by the exclusive administration of cyclophosphamide (without a therapeutic scheme) is classified as a late-onset nausea.[56] Thus, ondansetron was not required to prevent this effect.[56] A decrease in effectiveness is another reason for not using ondansetron prior to cyclophosphamide.[57]

This reduction in efficacy occurs because ondansetron is an inhibitor of CYP2B.[58] This occurs because cyclophosphamide is a prodrug that must be activated by CYP2B, resulting in 4-hydroxycyclophosphamide and aldophosphamide. These metabolites are transported to the site of action, where they undergo spontaneous cleavage, producing phosphoramide mustard, responsible for the pharmacological effects of the drug.[58]

Moreover, dexamethasone was added prior to chemotherapy, as a prophylactic agent to anaphylactic shock and also as an antiemetic.[59] Preferably, dexamethasone should be administered so that its peak coincides with the peak of physiological corticosteroids, which normally occurs at 8 a.m. and 16 p.m.[58] A peak in dexamethasone plasma concentration occurs within 60 min, and its action begins in 30 min.[55,60] Thus, the administration of dexamethasone should be started in the morning, 30 min before the administration of cyclophosphamide, preferably at 7:30 a.m.[58]

After the emesis caused by cyclophosphamide was classified as a delayed-type,[56] and in view of the decrease of cyclophosphamide efficacy when ondansetron is administered prior to this chemotherapeutic agent, ondansetron (8 mg PO[31,61] administered at 6 and 14 or 8 and 16 h post-ChT, and with a maximum dose of 16 mg after chemotherapy, not exceeding 32 mg per day) was the last drug used for the prophylaxis of emesis caused by cyclophosphamide.[31]

Other important adverse effects of cyclophosphamide include hematologic toxicity,[18] kidney failure,[20] hyponatremia,[45,62] neurological impairment,[45] amenorrhea,[18] early menopause,[46] hair loss,[3] hepatotoxicity (rare),[3] and late-onset cancer.[18] The dose of cyclophosphamide for the treatment of systemic lupus erythematosus, including those patients with neuropsychiatric and/or hematologic disorders, with class IV lupus nephritis, and with other serious manifestations of systemic lupus erythematosus, is 0.5–1 g/m^2 IV monthly,[5,62] with dosage adjustment in patients with hematological toxicity and kidney failure.[20,63] Adverse hematologic reactions caused by cyclophosphamide are classified as serious, as they cause high morbidity.[19,64] NADIR is the minimum hematologic counting that must be observed to see if the patient may or may not embark on another chemotherapeutic cycle.[65] The main hematological tests include platelets, neutrophils, white blood cells and neutrophil counts. Neutropenia is defined as a decrease in absolute neutrophil count <1500 cells/μL.[65] The main causes of neutropenia include hematologic disorders, autoimmune diseases, infection, adverse drug reaction, chemotherapy, and radiotherapy.[65] Thrombocytopenia occurs when the platelet count is <100,000/mm^3.[65] In a patient with a platelet count <81,000/mm^3 concomitantly experiencing leukopenia, the treatment should be discontinued until the platelet count increase to 99,000/mm^3.[65] However, cyclophosphamide can be used in the treatment of refractory thrombocytopenic purpura, and in this case, among the hematologic disorders, thrombocytopenia, and microangiopathic hemolytic anemia are included. Leukopenia[10] is defined as a white cell count <3500/mm^3.[65]

Hepatotoxicity is an adverse reaction caused by cyclophosphamide that normally occurs at high doses.[3] When hepatotoxicity occurs at low doses, the reaction is classified as a rare, but serious, event.[19,64] Acute liver failure with low-dose cyclophosphamide (200 mg) was described in a case report of a male Chinese aged 48 years with progressive glomerulonephritis secondary to Wegener's granulomatosis, 24 h after the administration of cyclophosphamide.[3] The diagnosis of granulomatosis with polyangiitis was established by pathological examination and c-ANCA.[3] The patient was being treated with high-dose methylprednisolone, plasmapheresis, intermittent hemodialysis and low intravenous doses of cyclophosphamide.[3] Other factors that may be associated with hepatotoxicity have been excluded, including antifungals, HIV, and hepatitis B and C.[3] Alanine transaminase increased from 41 U/L to 336 U/L at the day of administration of 200 mg of cyclophosphamide; in the next day, a peak of 566 U/L was reached.[3] Two weeks later, the patient was treated with another pulse of 200 mg of cyclophosphamide (second dose), and the concentration of alanine transaminase reached 1253 U/L.[3] A liver biopsy was not possible, because the patient had a clotting disorder.[3] Another laboratory parameter to be monitored is sodium concentration. Hyponatremia is considered as an electrolyte disorder identified in clinical practice.[45] Although many cases are mild or relatively symptomatic, hyponatremia is regarded as a clinically important finding,

with high morbidity and mortality. Neurological symptoms of hyponatremia occur at sodium levels <125 mmol/L.[66] Among the complications of hyponatremia, one can mention central nervous system disorders such as seizures, and even a permanent damage to the central nervous system, and death.[66] Syndrome of inappropriate antidiuretic hormone secretion (SIADH) was described in a case report in which this syndrome was associated with the use of IV cyclophosphamide in a dose of 500–1000 mg/m^2, with a serum sodium level <120 mmol/L in a patient presenting neurological complications.[62]

Cyclophosphamide may also cause cardiotoxicity. Routinely, echocardiography, a noninvasive method, is used to monitor cardiovascular function in patients treated with immunosuppressants.[36] Other non-invasive methods most widely used are electrocardiography and 24-h (ECG) Holter monitoring.[36] Invasive methods such as scintigraphy, magnetic resonance imaging and cardiac biopsy are also procedures that can be used in cases of adverse reactions classified as serious.[36]

Other adverse reactions caused by cyclophosphamide include amenorrhea, menopause, and late-onset cancer, including esophageal, lung and bladder adenocarcinoma.[18,46] Cyclophosphamide was administered at a dose of 750–1000 mg/m^2 body surface in LUMINA (Lupus in minorities, nature versus nurture), a multicenter study performed including 567 women aged under 51 years.[46] A decrease in gonadal function was found, and gonadal failure was defined as the occurrence of amenorrhea for more than six months without a history of hysterectomy.[46] Cyclophosphamide has also been associated with teratogenicity.[18]

As to cyclophosphamide dose adjustment in patients with kidney failure, this is only done in severe cases, that is, with a creatinine clearance <10 mL/min. The therapeutic regimen of cyclophosphamide should be modified, its dose should be reduced by 25%, and supplemented with 50% after dialysis.[34]

The dilution of the drugs used in cyclophosphamide routine was carried out with the goal of maintaining the highest possible concentration of the drug in its non-ionized form.[58] Thus, two parameters were taken into account: drug compatibility with the vehicle (saline, dextrose or ringer lactate) and its stability time in the vehicle; preference was given to those drugs whose stability time was longer, in order to ensure a more effective treatment.[33]

When the drugs used have proved compatible and stable with the same vehicle, preference was given to the choice of the same vehicle, as this facilitates their administration by the nursing staff and prevents pharmacokinetic interaction, which would cause ionization of the drugs and a decrease in their efficacy.[67]

Another important aspect that should be addressed is the education of the patient on the treatment to what he/she is being submitted. A study in the city of Natal with 40 women diagnosed with systemic lupus erythematosus at the Hospital Onofre has shown that patients showing greater adherence were those who understood correctly the treatment and also the disease; thus, these women satisfactorily understood the adverse events and the clinical management, in order to minimize these adverse drug reactions.[68]

Publications on adverse events are important tools for drug safety monitoring after their release for marketing purposes by the health authorities of each country.[69] This review of medication administration intended to standardize the administration of cyclophosphamide by health professionals, in order to minimize the adverse events caused by drugs, the so-called adverse drug reaction.[19]

Conclusion

The routine was developed in order to increase the area under the curve (AUC) for cyclophosphamide, and to attain an appropriate clinical management to minimize the adverse drug reactions that, if not properly prevented, will cause damage to the patient, for instance, an increased prevalence of vomiting, and acrolein deposition in the bladder. The clinical management of adverse reactions that alter laboratory tests was described in order to guide the application of these tests. Adverse reactions, even being rare, were highlighted because these events can cause high morbidity. Thus, the rational use of cyclophosphamide increases the safety of the treatment and reduces the cost of administration of this chemotherapeutic agent, since our goal is to avoid error.

Conflicts of interest

The authors declare no conflicts of interest.

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