

Regulation of the expression of somatic H1 subtypes in human lines

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Master's thesis / Diplomski rad

2022

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Rijeka / Sveučilište u Rijeci**

Permanent link / Trajna poveznica: <https://um.nsk.hr/um:nbn:hr:193:547640>

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UNIVERSITY OF RIJEKA

DEPARTMENT OF BIOTECHNOLOGY
GRADUATE PROGRAMME
DRUG RESEARCH AND DEVELOPMENT

Laura Bilaver

REGULATION OF THE EXPRESSION OF SOMATIC H1
SUBTYPES IN HUMAN CELL LINES

Master thesis

Rijeka, 2022.

SVEUČILIŠTE U RIJECI

ODJEL ZA BIOTEHNOLOGIJU
DIPLOMSKI STUDIJ
ISTRAŽIVANJE I RAZVOJ LIJEKOVA

Laura Bilaver

REGULACIJA EKSPRESIJE SOMATSKIH PODTIPOVA H1
U LJUDSKIM STANIČNIM LINIJAMA

Diplomski rad

Rijeka, 2022.

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**The master thesis was defended on _____ in/at
_____, before a committee
composed of:**

1. _____

2. _____

3. _____

Aknowledgment

I would like to express my special thanks of gratitude to my mentors dr.sc. Alicia Roque Cordova and Inmaculada Ponte Marull, who gave me the opportunity to work on this project. I want to thank them on the given advice, great understanding and professional relationship.

Secondly, I would also like to thank my parents for the support and trust and everything they gave me during my studies.

Abstract

Histone H1 or linker histone is involved in the regulation of chromatin structure (4). Histone H1 is part of a multigene family encoding eleven subtypes, seven somatic subtypes, and four germ-line specific subtypes (1). The complement of histone H1 is defined as the subtype composition and their proportions in a cell on a given condition. H1 complement is variable and several studies have reported that is altered in disease, in particular in cancer (2). Therefore, more studies must be performed in order to understand its functions in diseases. In this study the global aim is to understand regulation of the expression of somatic subtypes in human cell lines. Our first objective was to study the role of m6A in the regulation of the mRNA and protein levels of histone H1 subtypes. We used *cycloleucine* to inhibit METTL3, m6A methyltransferase. We tested several doses finding that in high doses of inhibitor prevent the ability of cells to replicate in the same hour period as untreated cells and effect of *cycloleucine* is dose-dependent in both cell lines. In HEK293T *cycloleucine* had shown different effects on the cell cycle depending on the dose and the time of treatment. In contrast, in HeLa the distribution of the cell cycle phases was not affected at 24h in any dose. H1 subtypes were differentially affected. At the mRNA level, HeLa cell line showed alteration of mRNA levels that occur in most of the H1 genes and controls at all doses tested, including the lowest dose. At the protein level, changes observed (increase or decrease) are in agreement with changes observed in the mRNA, except for H1.4. Considering the magnitude of the change, there is no correspondence between mRNA and protein levels. In general, the effects are different among subtypes supporting the idea that m6A plays a subtype specific role in H1 regulation. Our second objective was to study the regulation of H1 protein levels by the proteasome in HeLa and HEK293T. We used to inhibitors MG132 and bortezomib. We confirmed that the proteasome is involved in the degradation of H1 subtypes in a subtype- and cell-type specific manner.

Keywords: Histone H1, m6A regulation, proteasome degradation

Sažetak

Histon H1 ili vezni histon sudjeluje u regulaciji strukture kromatina (4). Histon H1 dio je multigenske obitelji koja kodira jedanaest podtipova, sedam somatskih podtipova i četiri podtipa specifična za zametnu liniju (1). Komplement histona H1 definiran je kao sastav podtipa i njihov udio u stanici u danom stanju. H1 komplement je varijabilan i nekoliko je studija objavilo da se mijenja tijekom bolesti, posebice kod raka (2). Stoga je potrebno provesti više studija kako bi se razumjele njegove funkcije u bolestima. U ovoj studiji globalni cilj je razumjeti regulaciju ekspresije somatskih podtipova u ljudskim staničnim linijama. Naš prvi cilj bio je proučiti ulogu m6A u regulaciji razine mRNA i proteina podtipova histona H1. Koristili smo cikloleucin za inhibiciju METTL3, m6A metiltransferaze. Testirali smo nekoliko doza otkrivši da visoke doze inhibitora sprječavaju sposobnost stanica da se repliciraju u istom vremenskom razdoblju kao i netretirane stanice, a učinak cikloleucina ovisi o dozi u obje stanične linije. U HEK293T cikloleucin je pokazao različite učinke na stanični ciklus ovisno o dozi i vremenu liječenja. Nasuprot tome, u HeLa distribucija faza staničnog ciklusa nije bila promijenjena u 24 sata ni u jednoj dozi. Podtipovi H1 različito su pogođeni. Na razini mRNA, stanična linija HeLa pokazala je promjenu razina mRNA koja se javlja u većini H1 gena i kontrola pri svim testiranim dozama, uključujući najnižu dozu. Na razini proteina, uočene promjene (povećanje ili smanjenje) u skladu su s promjenama uočanim u mRNA, osim za H1.4. S obzirom na veličinu promjene, ne postoji podudarnost između razine mRNA i proteina. Općenito, učinci su različiti među podtipovima što podržava ideju da m6A igra specifičnu ulogu podtipa u regulaciji H1. Naš drugi cilj bio je proučiti regulaciju razine H1 proteina pomoću proteasoma u HeLa i HEK293T. Koristili smo inhibitore MG132 i bortezomib. Potvrdili smo da je proteasom uključen u razgradnju podtipova H1 na način specifičan za podtip i tip stanice.

Ključne riječi: Histon H1, regulacija m6A, razgradnja proteasoma

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1. Introduction

1.1 Histone H1

Nucleosomes are the structural units of eukaryotic chromatin. The major protein component of chromatin is histones. They are divided into core and linker histones (3). In the last few years, researchers have focused more on core histones and their role in gene regulation. In contrast, linker histone, also known as histone H1, is not fully understood.

In humans, histone H1 is part of a multigene family encoding eleven subtypes, which are grouped into seven somatic subtypes, and four germ-line specific subtypes (1). Somatic subtypes of H1 include H1.0 to H1.5 and H1X, whereas germ-line specific subtypes include from H1.6 to H1.9. Regarding the structure of histone H1, it is known that it has 3 structural domains: one structured globular domain and two disordered N-terminal and C-terminal domains. The average level of disorder in histone H1 sequences equivalents to 65.42%, whereas up to 100% of disordering corresponds to amino acids that cover its terminal domains. Furthermore, it was reported that histone H1 subtypes are enriched in disorder promoting amino acids. Function of histone H1 is determined by localization of recognition motifs, disordered stretches and the binding sites in all domains, suggesting that a state of intrinsic structural disorder might direct the histone H1 activity governed by specific interactions with DNA and partnering proteins (4).

Histone H1 or linker histone is involved in the regulation of chromatin structure. It has been reported that the knockout or knockdown of one or two linker histones, can be compensated in the terms of the total H1 content, through up-regulation of the remaining H1 genes (5). Moreover, studies revealed that inactivation of three subtypes lead to 50% of the normal amount of H1 and resulted in lethality in mice, indicating importance of accurate level of H1 deposition on chromatin for mammalian

development (6). In human, somatic subtypes from H1.1 to H1.5 are encoded in chromosome 6, in the major histone cluster, which is located in the self-organizing compartment called histone locus body (HLB). The remaining two somatic subtypes, H1X and H1.0, are encoded in chromosomes 22 and 3, respectively. They are known as replication independent subtypes because their transcription is uncoupled from histone locus body transcription (1). There are multiple copies of genes coding for core histones, while genes that are encoding for histone H1 subtypes are single-copy genes (5). Generally, histones are a target for many post-translational modifications such as, methylation, ubiquitylation, phosphorylation and acetylation. These modifications can have impact on histone interactions with DNA or with each other. Likewise, some modifications provide a docking site for specific readers, for instance chromatin remodeling complexes containing bromo domains that have affinity for acetylated histones (7). That said, histone post-translational modifications and chromatin remodeling proteins play important role in transcription, replication, and DNA repair.

The complement of histone H1 is defined as the subtype composition and their proportions in a cell on a given condition. H1 complement can vary, it depends on cell cycle phase, time of the development, and on the cell type. Several studies have reported that H1 complement is altered in disease, in particular in cancer (2). However, histone H1 regulation in physiological conditions and in disease is not fully understood. Therefore, more studies must be performed in order to understand its functions in diseases. Recently, scientists have revealed that in ovarian adenocarcinoma cancer cells show a 40% reduction in overall linker histone mRNA level compared with benign tumors (2). Another example, under genotoxic stress, CHD8 recruited the linker histone to the p53 promotor, which caused chromatin condensation. p53 is important tumor suppressor gene. With p53 chromatin condensation, its transcriptional activity is repressed, as a result p53 functions are inhibited and the possibility of cell tumorigenesis is increased

(2). In Alzheimer's disease one of the most important characteristics are amyloid plaques in neurons. Since the discovery that the linker histone is present in amyloid plaque (2), it raised more interest to investigate relationship between the histone H1 and pathogenesis of Alzheimer's disease. Additional investigation revealed that H1 can change the conformation of its own C terminal domain into an all β structure in the presence of detergents, which is ready for forming ribbon-like fibers (2). Another study revealed that linker histone can interact with β -amyloid peptide, this interaction consequently causes both protein conformation changes. Altogether, these studies suggest that the linker histones can form the amyloid-like fibers themselves. Likewise, H1 can also change the conformation of beta-amyloid fibrils and facilitate the aggregation of these amyloid fibrils (2).

Histone H1 combined with nucleosomes forms the chromatosome, which can compact the chromatin into higher-order forms (3). This complex has an important role in controlling cellular processes that are essential for chromatin modulation in a specific manner, such as regulation of cell division, gene expression, DNA damage response and cell fate. Chromatin modulation that is nucleosome dependent is influenced by the regulation of histone H1 at different levels: transcription, post-transcriptional processes and mRNA stability, translation efficiency and protein stability and post-translational modifications. This processes provide broad spectrum for H1 regulation to modulate the structure and function of the chromatin (8). In this work I will focus on two aspects of H1 regulation: the post-transcriptional regulation mediated by m6A modification (m6A-epitranscriptome) and in the regulation of the protein levels by proteasomal degradation.

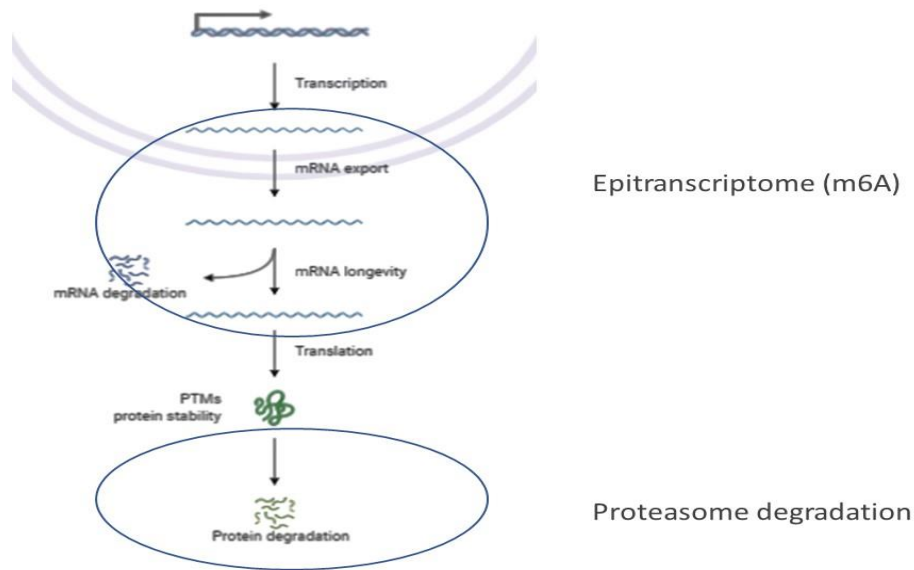


Figure 1. Critical steps in the regulation of the mRNA and protein levels (original figure made with biorender)

1.2. m⁶A regulation

There are plenty types of documented epigenetic modifications, such as chromatin remodeling, DNA methylation, histone modification and noncoding RNA modification, among which, the methylation modification of DNA and RNA are extremely important (9). In 1974., N6-methyladenosine (m⁶A) was discovered as the most abundant modification of mRNA in the majority of eukaryotes (6). So far, there are over 100 kinds of RNA modifications identified in many types of RNAs, including mRNA, tRNA, rRNA, snRNA, microRNA, and long non-coding RNA (lncRNA). m⁶A is present in 0.1–0.4% of all adenosines in global cellular RNAs and accounts for almost 50% of all methylated ribonucleotides (10). Until recently, very little was known about its functional significance and the extent of transcript identities. m⁶A is one of the main epigenetic markers of RNAs and essentially occurs in two consecutive sequences G m⁶A C (~70%) and A m⁶A C (~30%)(11). RNA m⁶A modification plays important role in RNA translation, stability, translocation, export, splicing, and high-level structure. The analysis of the m6A transcriptome by next generation

sequencing, revealed that approximately 3-5 m⁶A modifications occur in every mRNA out of one third of total mammalian mRNAs (5). The main post-transcriptional modification of mRNA is m⁶A, which mostly appears in RRACH sequence. Likewise, in other RNA types, m⁶A regulates mRNA at different levels, including structure, maturation and degradation. It is a reversible modification regulated by two important catalytic proteins, methyltransferases (writers) and demethylases (erasers) (12). The specific functions of this modification are mediated by the m⁶A binding proteins (readers).

1.2.1. m⁶A “writers”

m⁶A is incorporated by methyltransferase complex that consists of METTL3, METTL14, Wilms Tumor 1 Associated Protein (WTAP), KIAA1429, METTL16, RNA Binding Motif Protein 15 (RBM15), and zinc finger CCCH domain-containing protein 13 (ZC3H) (12). METTL3 was the first discovered methyltransferase, it plays important role in m⁶A methylation and recent studies have shown that its expression could alter the total m⁶A methylation level (13). In addition, it acts as the catalytic core, transferring methyl group from S-adenosylmethionine (SAM) to adenine. METTL14 forms a complex with METTL3 that methylates RNA substrates that have a GGACU domain. Apart from that, METTL14 supports METTL3 in recognizing special RNA substrates (10). WTAP doesn't have methyltransferase activity but binds to complex with METTL3-14 to affect m⁶A methyltransferase activity *in vivo* and promotes m⁶A in nuclear speckles (6). Additionally, it can bind unknown factors to the methyltransferase complex and modulate methylation (10).

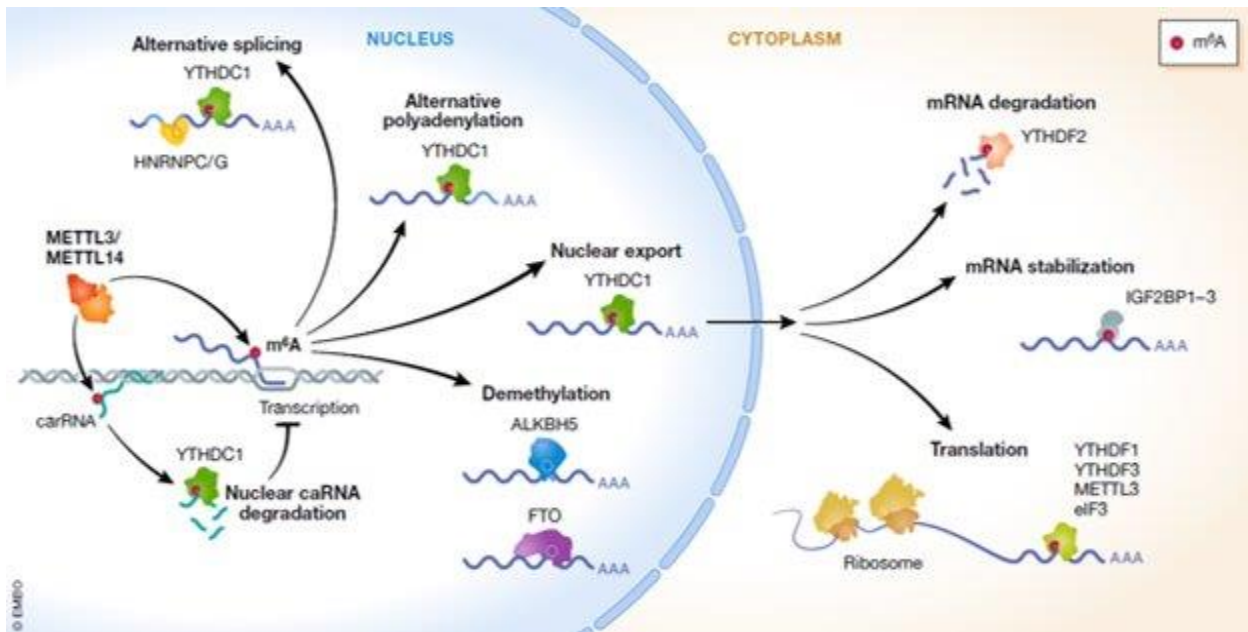


Figure 2. Role of m6A in the regulation of gene expression (14)

1.2.2. m6A “erasers”

The demethylases, also known as “erasers” remove the m6A modifications in RNA. Demethylation is achieved by two main m6A eraser proteins: fat mass and obesity-associated protein (FTO) and AlkB homolog 5 (ALKBH5) (13). FTO was the first discovered demethylase, its role is to modulate alternative splicing as well as the 3'-end mRNA processing in 293T cells. The other demethylase identified so far is ALKBH5. ALKB domain (alpha-ketoglutarate-dependent hydroxylase) is located in the middle regions of both FTO and ALKBH5. It consists of two active motifs, which bind iron Fe (II), α -ketoglutarate (α -KG) and substrate. ALKBH5 domain has an additional A motif on N-terminal responsible for localizing ALKBH5 at nuclear speckles. So far it has been reported that FTO accumulates in adipose and cerebral tissue, while ALKBH5 is more expressed in testes (15).

1.2.3. m6A “readers”

Specific group of proteins known as “readers” recognize and potentially bind RNA to regulate its downstream functions, such as mRNA stability, splicing,

mRNA structure, translation efficiency and many others (9). Depending on the reader, different m⁶A functions have been found. The most distinguished “reader” is YTH domain family proteins which include YTHDF1, YTHDF2, and YTHDF3 in the cytoplasm, and YTH domain containing 1 (YTHDC1) in the nucleus. Recent studies have shown that YTHDF1 domain promotes the translation of m⁶A-methylated mRNA, YTHDF2 advances the degradation of m⁶A-methylated mRNA whereas YTHDF3, together with YTHDF1 and YTHDF2 domain enhances the metabolism of m⁶A in cytoplasm (12). On the other hand, another example of mRNA-binding protein is insulin-like growth factor 2 (IGF2BP) family members. They are single-stranded RNA-binding proteins (RBPs), which contain six canonical RNA-binding domains, two RNA recognition motif (RRM) domains, and four K homology (KH) domains. IGF2BPs targets mRNA transcripts by recognizing consecutive GG(m⁶A)C sequence. It promotes mRNA stability by binding to target transcripts and therefore affects gene expression outputs. Depending on the reader binding protein different functions can be performed depending on a different cellular context (16).

	m⁶A "Readers"	Function
YTH family proteins	YTHDF 1	Promote translation of m ⁶ A-methylated mRNA
	YTHDF 2	Advances the degradation of m ⁶ A methylated mRNA
	YTHDF 3	Together with YTHDF1 and YTHDF2 domain enhances the metabolism of m ⁶ A in cytoplasm
Family of RNA-binding proteins	IGF2BPs	Promotes mRNA stability, affects gene expression outputs, targets mRNA transcripts by regognizing GG(m ⁶ A)C sequence

Table 1. Summarized function of the m⁶A “readers”

1.3. m⁶A methylation in cancer

Currently, m⁶A methylation has been found to have an influence in biological regulatory functions in cancer initiation. Recent studies revealed that m⁶A methylation has impact on tumor initiation and progression through several mechanisms (6). It has been reported that METTL3 and oncogene CDCP1 are up-regulated in bladder cancer and that way correspond with its progression status. METTL3 elevated m⁶A level of CDCP1, hence promoting its translation regulated by YTHDF1 (17). AF4/FMR2 is a family of transcriptional activators that acts as a direct upstream regulator of MYC and can increase MYC expression. Moreover, METTL3 promotes the expression of both MYC and AFF4. Inhibition of METTL3 has revealed that prevents bladder tumor cell proliferation, invasion, migration and survival *in vitro* and affects cell proliferation *in vivo* (17).

Furthermore, METTL3 is associated with HBXIP (hepatitis B X-interacting protein), and its expression is increased in breast cancer. Studies revealed that METTL3 enhances the malignant phenotypes of breast tumor. METTL3 is also known as promotor of HBXIP expression. HBXIP also facilitates METTL3 expression by inhibiting miRNA let-7g (tumor-suppressor), which reduces METTL3 expression through targeting its 3' UTR (6).

1.4. Proteasome degradation

Proteasomal degradation can be divided into two different types. Ubiquitin-dependent degradation (26S) and ubiquitin independent degradation.

Proteasome inhibitors present important class of drugs for the treatment of mantle cell lymphoma and multiple myeloma. So far they are still in clinical trials for additional types of cancer (18). Another role of proteasome inhibitors are bone resorption inhibition, immunosuppressant functions and other applications. Bortezomib was the first proteasome inhibitor approved by US Food and Drug Administration (FDA) in 2003 (18). Primary

mechanism of Bortezomib is inhibition of catalytically active subunits of the proteasome.

Ubiquitin dependent on proteasome (UPP) pathway is the most prevalent way by which most intracellular proteins are degraded. However, extracellular proteins and some cell surface proteins are degraded in lysosomes or taken up by endocytosis (19). The proteasome consists of two subcomplexes known as 26S unit that represents the major proteasome. This degradation pathway requires ATP, it has three subunits, catalytic core particle known as 20S proteasome (approximately 700 kDa) with peptidase activity and one or two 19S regulatory particles (approximately 900 kDa). Proteasome structure contains two groups of 7α subunits and two groups of 7β subunits which are located on the outside of the 20S subunit and give a cylindrical or barrel shape to the structure. The α subunit represents a gate to 20S proteasome, whereas β subunit has three peptidase activities, caspase-like, trypsin-like and chymotrypsin-like activities to directly degrade proteins (20). The 19S regulatory caps are responsible for ubiquitin recycling and substrate unfolding.

Ubiquitin-proteasome pathway is a way of a cell to control misfolded or damaged proteins. In order for proteins to be degraded they have to be recognized by the proteasome, and that involves tagging with small ubiquitin (Ub) molecule. Binding of Ub molecule on a protein to mark them for degradation requires three enzymatic components. Components consists of three enzymes E1 which is Ub activating enzyme and E2 which carries ubiquitin molecule and prepares it for conjugation. E3 is the key enzyme in this process, it is a protein ligase that recognizes specific substrate and catalyzes the transfer activated Ub molecule to substrate. Once protein is tagged, it is recognized by 26S proteasome, a large multicatalytic protease complex that degrades ubiquitinated proteins to small peptides (8). After binding of ubiquitin molecule to substrate ubiquitination chain is formed, meaning that on the same ubiquitination site subsequent events are repetitively occurring. In contrast to other regulatory

mechanisms protein degradation is irreversible. Many transcription factors are ubiquitinated and degraded by the proteasome. Binding of ubiquitin molecule affects transcription by multiple mechanisms.

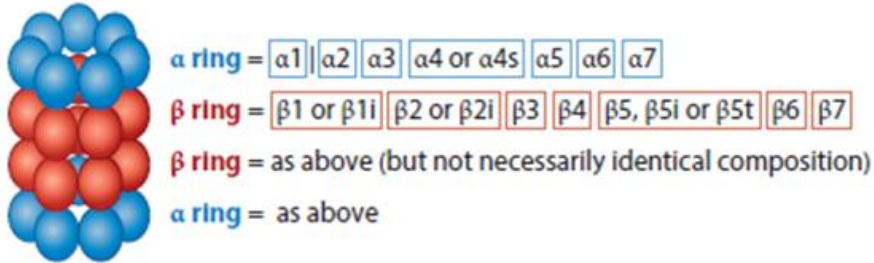
Moreover, apart from this ubiquitin-dependent pathway there is another mechanism that does not require ubiquitin molecule for proteasomal degradation. The 20S core particle along with regulatory complexes function as proteasome activators. Proteasome activator 19S, known as PA700 consists of 19 or more protein subunits, its functions are removal of ubiquitin chains, recognition of ubiquitin tagged proteins, the unfolding and translocation of proteins in the 20S core. Apart from this, the 19S complex is responsible for recognition of non-ubiquitinated proteins. Proteasome activator 11S, known as PA28, PA26 and REG is made of seven subunits. PA28 $\alpha\beta$ is composed of α and β subunits, whereas PA28 γ holds 7 γ subunits. The 11S is not able to unfold proteins because it does not recognize ubiquitin or use ATP. PA200 is a monomeric protein, approximately 200kDa, which does not bind Ub molecule or uses ATP. Binding of a different regulatory subunits on to 20S core can lead to hybrid forms with different properties. The 26S proteasome, containing 20S core with two 19S regulatory domains is a common form that requires ATP and is ubiquitin-dependent. On the other hand, the 20S proteasome alone or bonded with 11S and/or PA200 cleaves peptides and misfolded proteins (18).

20S can degrade unfolded proteins and proteins containing intrinsically disordered regions (21). As Histone H1 has two domains that are intrinsically disordered the group have shown that this mechanism could function in the case of H1 degradation, and it is dependent on the C-terminal domain.

Despite the most common proteasome targeting for degradation is ubiquitination, at the moment it is not known whether H1 is degraded with that mechanism. So far it is known that the proteasome is involved in the regulation of H1 protein levels in T47D and that H1 can be degraded without ubiquitination by *in vitro* assays. In this study we are trying to establish the

role of the proteasome in H1 regulation is a common feature in human cell lines.

20S proteasome core particle



26S Proteasome

Other proteasome forms with different regulatory caps

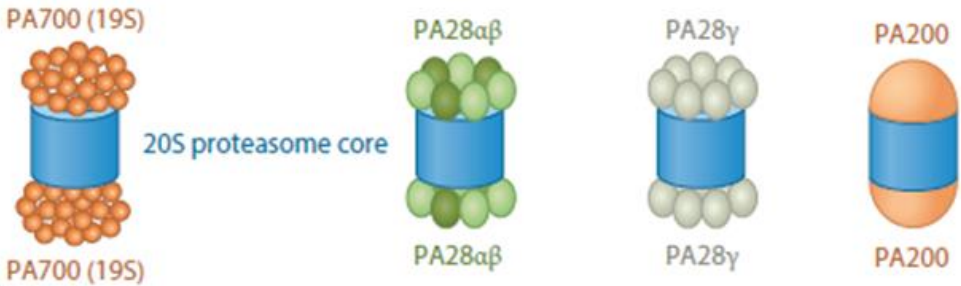


Figure 3. Different types of proteasomes (18)

2. Aim of the study

The global aim of this study is to understand Histone H1 subtypes regulation. I focused on the role of m6A in H1 regulation and in the role of the proteasome in the degradation of H1 subtypes. In this context, the specific objectives of this work were:

- 1) Analysis of the effect of METTL3 inhibition in cell growth and cell cycle
- 2) Analysis of the effect of METTL3 inhibition in the mRNA and protein levels of histone H1 subtypes
- 3) Analysis of the accumulation of H1 subtypes after proteasome inhibition in HeLa and HEK293T cells

3. Materials and methods

3.1. Cell lines

In this study two different cell lines were used HeLa and HEK293T. The HeLa cell line represents epithelial cells isolated from a cervical tissue sample obtained from a patient diagnosed and treated for terminal cervical cancer. Cells were cultured in DMEM media. The HEK293T cell line is isolated from human embryonic kidney HEK293 cells. Both cell lines were maintained in DMEM media.

Dulbecco's modified Eagle's (DMEM) media contains 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate.

All cell lines were grown at 37°C and 5% CO₂.

3.1.1. Treatment with *Cycloleucine*

The first step was to calculate the amount of drug and weigh it. Afterwards, the drug was dissolved in an DMEM medium and sterilized. Sterilization was carried out by filtration. After all the drug was dissolved in the medium, it was passed through a 0.22 µm filter into a new tube. 3 ml of medium were removed from the plate where the cells were located and a new 3 ml containing *Cycloleucine* were added and left in the incubator at 37°C with 5% CO₂ for duration of the treatment. The treatment was performed using a final concentration of the inhibitor of 50, 75, and 100mM and incubating the cells for 12 or 24h.

3.1.2. Treatment with MG132 and Bortezomib

This treatment was similar to the one mentioned above. The first step was to calculate the amount of drug needed from the stock solution to achieve a final concentration in the plate of 20µM forMG132 and 20nM for bortezomib. Both drugs were dissolved in DMSO, so to the control plate a

similar amount of DMSO was added. 3 ml of medium were removed from the plate with the cells and a new 3 ml containing MG132, bortezomib or DMSO were added and left in the incubator at 37°C with 5% CO₂ for 12 hours. The treatment was performed with both cell lines.

Cell growth analysis:

HeLa and HEK293T cells were plated at 0.1 million cells/mL and grown in their usual conditions and in the presence of 50, 75 and 100 mM *cycloleucine*. Cells were harvested at 24 and 48h and counted with a TC20 automated cell counter. The number of cells in each condition was plotted to obtain a growth curve. All the measurements were performed by triplicate.

3.2. Flow cytometry

Flow cytometry is a technique that provides fast analysis of single cells in solution. Flow cytometer uses lasers as light sources to produce both scattered and fluorescent light signals that are read by detectors. These signals are transformed electronic signals and analyzed by a computer. Depending on their light cell characteristics can be analyzed (18).

To determine effect of *Cycloleucine* on cell cycle Cytometry analysis was performed. In order to prepare the samples for cytometry cells were collected from P100 plates, transferred to a 15mL Falcon tube and counted. Trypan blue was used for cell counting. Further, a 1:1 mix of dye and cell suspension was made, resuspended, and counted with TC20 automated cell counter. Following this, approximately 1 million cells were fixed in 70% ethanol, propidium iodide was added to a concentration of 1mg/ml and frozen on -20°C. Propidium iodide was used for staining, cytometry analysis was performed at the Cell Culture Facility of UAB.

3.3. Gene expression analysis

Method used for analysing a gene expression in human cell lines was reverse transcription quantitative PCR (RT-qPCR).

3.3.1. RNA extraction

To prepare samples for this analysis total RNA was isolated using protocol from The PureLink RNA Mini Kit [PureLink \(Termofisher\)](#). The concentration and quality of the samples were determined using Nanodrop.

3.3.2. Reverse transcription

After concentration and quality calculation, 100ng of RNA were retrotranscribed using random priming and the protocol from Invitrogen SuperScript Reverse Transcriptase (RTs). Reaction mixtures were put in a PCR machine where samples are converted to cDNAs using the conditions suggested by the manufacturer (Invitrogen).

3.3.3. RT-qPCR

In quantitative PCR, reactions are characterized by the point in time during cycling when the amplification of a target is first detected and not the amount of target accumulated after a fixed number of cycles.

Additionally, genes used in analysis included all H1 somatic subtypes expressed in the specific cell line, cell cycle genes, m6A-metabolism genes, and housekeeping genes. Histone H1 somatic subtypes expressed in HeLa are H1.0, H1.1, H1.2, H1.4, H1.5, and H1X. In HEK293T, we found five H1 subtypes expressed: H1.0, H1.2, H1.3, H1.4, and H1X. The rest of the genes selected for the analysis were *myc*, *β -actin*, *kif4*, *igf2bpi*, *brca1*, *mettl14* and *fto* (**Pogreška! Izvor reference nije pronađen.**)

To determine the effect of *cycloleucine* the difference between the Cq values for each gene versus the untreated sample were expressed as Fold change. All the measurements were carried out by triplicate. Cq's with differences greater than 0.5 cycle were discarded.

HEK293T and HeLa			
Gene	Primer sequence (Forward)	Primer sequence (Reverse)	GENE ID
H1.0	CATGATCGTGGCTGCTATCCAG	GTTCTCACCCACCTTGTAGTGG	14958
H1.1	AAGGCACACTGGTGCAGACCAA	CGGATGCTTTCGCCTTCACTGA	80838
H1.2	TGGTGTGCCACACCCAAAAAG	GGCTTCTTAGGTTTGGTTCCGC	50708
H1.3	AAGGCGACTGGTGTGCCACA	TTGGCTGGACTCTTTGCTGCCT	14957
H1.4	CCGAACCTCATCACCAGGCTGT	TACCCTTGCTCACCAGGCTCTT	50709
H1.5	GTTGCCAAGAGTCCTAAGAAGGC	AGCGGTCTTAGGCTTGGTAACC	56702
H1.X	AGAACCAGCCAGGCAAGTACAG	TTCTGCTGGTCGAACCATGCCA	243529
MYC	TCGCTGCTGTCTCCGAGTCC	GGTTTGCCTCTTCTCCACAGAC	17869
BACTIN	CATTGCTGACAGGATGCAGAAGG	TGCTGGAAGGTGGACAGTGAGG	11461
KIF4	GTATCGCCACAATTCTCGAAGCC	GCTCCTCAAACAGCATCTTCTGC	16571
IGF2BP1	CCTGGCTCATAACAACCTTCGTCG	CCTTACAGTGATGGTCCTCTC	140486
BRCA1	CGAGGAAATGGCAACTTGCCTAG	TCACTCTGCGAGCAGTCTTCAG	12189
METTL14	AGAGTGCGGATAGCATTGGTGC	CTCCTTCATCCAGACACTCCG	210529
FTO	GCCTCGGTTTAGTTCCACTCAC	GTCGCCATCGTCTGAGTCATTG	26383

Table 1. Primer sequence of genes used in RT-qPCR

3.4. Total protein extraction

For total protein extraction the initial material was a cellular pellet of approximately 1 million cells. First step was cleaning the cells with PBS (Phosphate Buffered Saline) and adding 100-200 μ L of Ripa buffer (Radioimmunoprecipitation assay buffer) that was supplemented with PIC (proteases inhibitor cocktails) and incubated on ice for 30 minutes. Next, cells were mechanically disrupted with syringe and centrifuged 30min at 13000rpm at 4°C. Supernatant was collected and quantified using Bradford.

3.5. Bradford protein assay

Bradford assay is a Coomassie dye-binding assay for protein quantification. The assay was performed at room temperature, then measured at 450nm and 595 nm following a short room temperature incubation. For protein quantification a standard curve prepared with BSA (Bovine Serum Albumin) dilutions at known concentrations was used.

3.6. Western blot

3.6.1. Composition of buffers

Running buffer 10x (1L) consists of 30.3g of TRIS, 138.9g Glycine and 10g of SDS. Running buffer (1L) 1x consists of 100ml of Running buffer 10x and 900ml of mQ water.

SDS-PAGE 12% Running gel consisted of 3.4ml of mQ water, 2.5 ml of Tris buffer pH 7.4, 4.5ml Acrylamide, 100 μ l 10% SDS, 100 μ l of PBS, 10 μ l of TEMED. Stacking gel consisted of 3.3ml of mQ water, 0.68ml of 1.5 pH Tris buffer, 0.83ml Acrylamide, 50 μ l 10% SDS, 50 μ l of PBS and 5 μ l of TEMED.

Transfer buffer 10x (1L) consisted of 58.2g TRIS, 29.3g Glycine and 4g SDS. Transfer buffer 1x (1L) consists of 100ml transfer buffer 10x, 100ml of methanol and 800ml mQ water.

For washing the membranes Tween TBS (TTBS) 1x0.1% TWEEN buffer was used. TTBS 1x consisted of 100ml TBS 10x, 900ml mQ water and 1ml TWEEN.

Blocking solution consists of 5g powder milk in 100ml TTBS.

3.6.2. Western blot procedure:

Materials used for Western blot analysis were PVDF transfer membrane, transfer buffer, wash buffer (Tris-buffered saline), blocking buffer, primary and secondary antibodies, and chemiluminescent reagents.

The first step was SDS-gel electrophoresis of proteins on 15% acrylamide gel followed by transfer at PVDF membrane on 100V for 1 hour. This was followed by blocking of membrane for 1 hour in blocking solution and incubation with primary antibody overnight (**Pogreška! Izvor reference nije pronađen.**) Next day, membrane was washed 3 times in wash buffer and incubated with the appropriate secondary antibody for 1 hour at room temperature. After incubation, the membrane was washed 3 times with

wash buffer and incubated with chemiluminescent developing reagents for 5 minutes. The last step was placing the blot in clear plastic wrap and imaging the blot with imaging system (Chemidoc). In all cases, tubulin was used as a loading control. Western blot images were quantified using BioRad Image Lab software.

Antibody	Supplier	Reference	Dilution	secondary Ab
Tubulin	Invitrogen/Fisher	scientific11839933	1:1000	Anti-mouse
β catenin	becton dickinson	610153	1:2000	Anti-mouse
H1.0	Thermofisher	PA5-51466	1:500	Anti-mouse
H1.2	Thermofisher	711909	1:500	Anti-rabbit
H1.3	Abcam	ab183736	1:1000	Anti-rabbit
H1.4	Thermofisher	702876	1:2500	Anti-rabbit
H1.5	Thermofisher	711912	1:1000	Anti-rabbit
H1.X	Abcam	ab31972	1:1000	Anti-rabbit

Table 2. Dilutions of the primary and secondary antibodies

4. Results

Previous results in the lab have shown that m6A is present in variable amounts on the transcripts of H1 subtypes. The next logical step is to determine the role of this modification in the mRNA and protein levels of H1 subtypes. For that reason, we used a chemical inhibitor of METTL3, *cycloleucine*.

4.1. Effects of *cycloleucine* treatment in cell growth

First were analysed different doses of the drug in two different cell lines HEK293T and HeLa and performed a growth curve (Figure 4a and 4b).

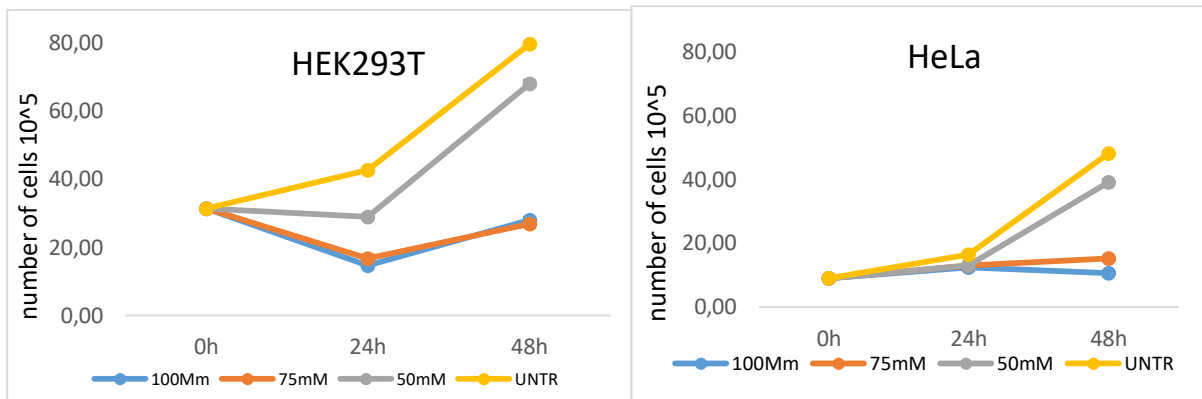


Figure 4. Growth kinetics of HEK293T (A) and HeLa (B) in the presence of *cycloleucine*. The cycloleucine concentration is represented with different colors according to the legend. The points represent the average of three measurements and the error bars correspond to the standard deviation of the values

HEK293T and HeLa cells were treated with three different doses 100mM, 75mM and 50mM, counted after 0h, 24h and 48h and compared with untreated cells.

The growth curve showed that the effect of *cycloleucine* is dose-dependent. High doses of inhibitor prevent the ability of cells to replicate in the same hour period as untreated cells. It is important to highlight that the lower dose (50 mM) does respect the duplication time of the cells.

4.2. Effects of cycloleucine treatment in cell cycle

As histone H1 transcript levels change during cell cycle, it is important to know if the inhibitor alters the distribution of the cell cycle phases. The changes in cell cycle upon *cycloleucine* treatment were analysed by Flow cytometry (Figure 5.).

In HEK293T, the addition of *cycloleucine* had different effects depending of the dose and the time of treatment. At 50mM, there is a decrease at 24h in S phase and accumulation of cells in G1 phase. At 75mM, there is a small accumulation of cells in S phase and G2M at 24h, coupled with a decrease in G1. At 100mM at 48h, we can see an accumulation of cells in G2M phase, coupled with a decrease of the percentage of cells in G2M phase. The later effect was also observed in HeLa at 48h in all the tested doses. In contrast with HEK293T, in HeLa the distribution of the cell cycle phases was not

affected at 24h in any dose. Taking into account that the inhibition affected the phases of cell cycle at 48h, in all doses and for both cell lines, the rest of the experiments were performed using 24h of treatment, where a minimum effect is observed.

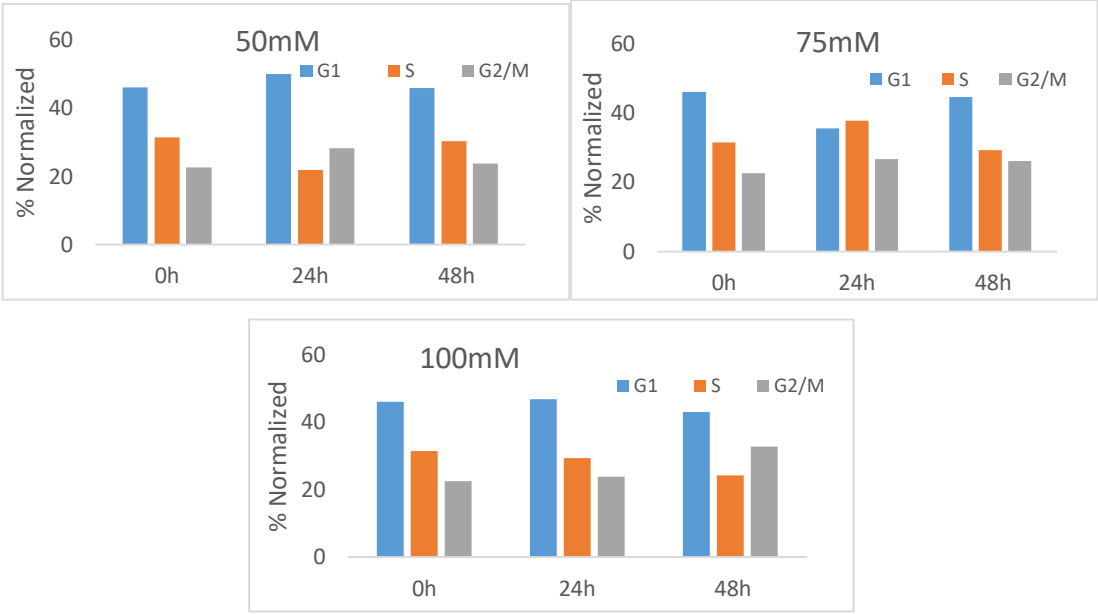


Figure 5. Effect of the treatment with *cycloleucine* on the cell cycle of HEK293T cells. The values correspond to the percentage of cells assigned to each phase of the cell cycle according to the propidium iodide fluorescence.

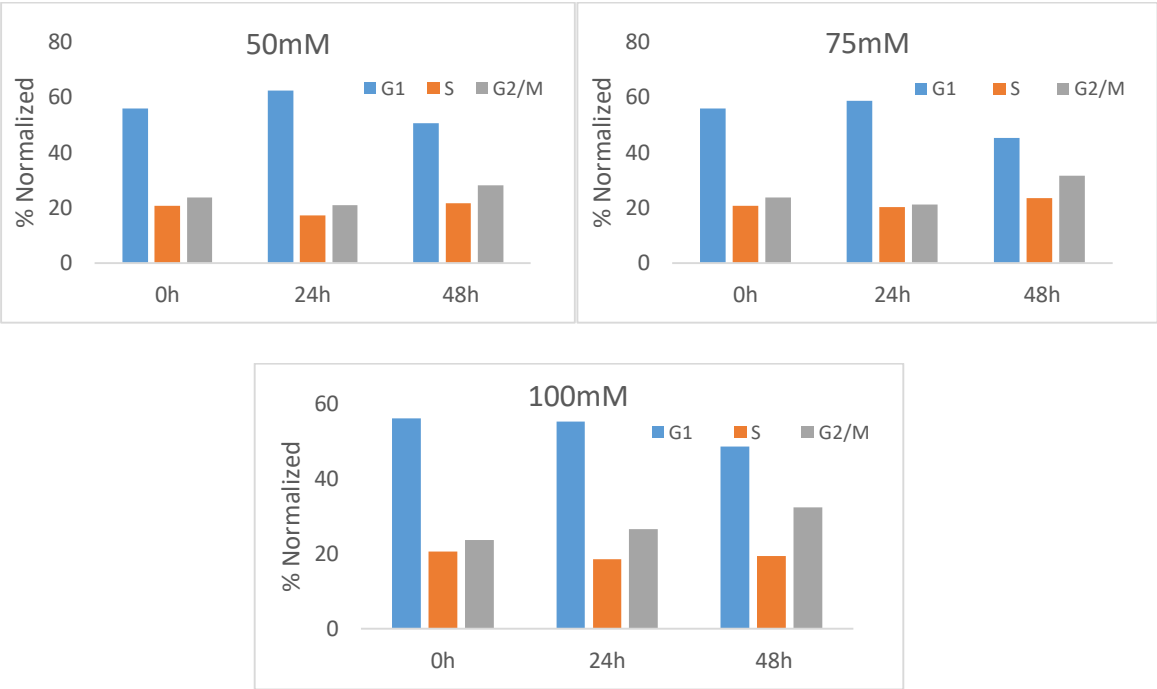


Figure 6. Effect of the treatment with *cycloleucine* on the cell cycle of HeLa cells. The values correspond to the percentage of cells assigned to each phase of the cell cycle according to the propidium iodide fluorescence.

4.3. Effect of METTL3 inhibition on histone H1 mRNA levels

In previous experiments we found that the mRNA of H1 subtypes contained m6A and the hypothesis is that this mark contributes to the regulation of their mRNA levels. To analyse the effect of METTL3 inhibition on H1 mRNA levels RT-qPCR was performed. Alongside H1, control genes and some cell-cycle related genes were used. Cell cycle related genes used were: MYC, BRCA1, some genes associated with m6A metabolism METTL14, IGF2BP1, and FTO, as well as a housekeeping gene β -actin.

Figure 7. shows results of RT-qPCR expressed as fold change in the HeLa cell line after 24h treatment with different doses of inhibitor.

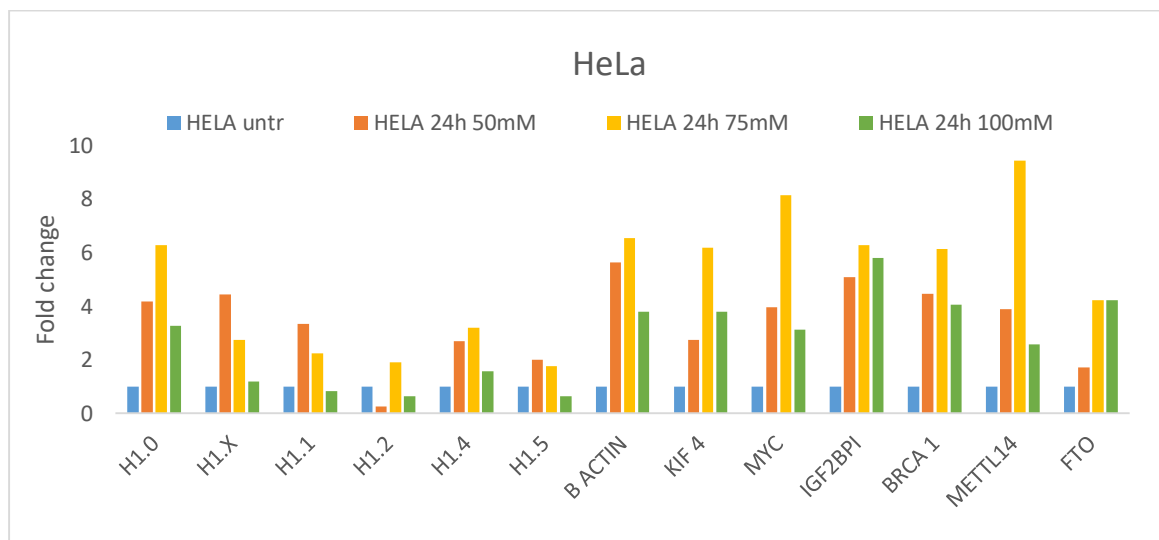


Figure 7. Quantification of the mRNA levels in HeLa after treatment with *cycloleucine* at different doses for 24h by RT-QPCR. The results are expressed as fold change of the untreated values.

Regarding the H1 subtypes, in general, and at doses of 50mM and 75 mM of inhibitor, we can observe an increase in mRNA levels, except in the case of H1.2, where a very clear decrease is observed at 50 mM. and an increase to 75 mM. At the 100 mM dose, only H1.0 and H1.4 maintain the mRNA

level above the control, H1.2 decreases, and in the rest of the subtypes the levels return to the initial values.

The subtypes, H1.2, H1.0, and H1.4, are the ones that present the most significant alterations in all doses of inhibitor. This is in agreement with the MeRIP data that indicates that the more methylated subtypes are H1.0, H1.4, and H1.2. Moreover, both cell cycle genes, MYC and BRCA1 show a behavior similar to the high methylated H1s. In the rest of the tested genes an increase was found in the mRNA levels of variable magnitude depending on the gene and dose of the inhibitor.

In general, we can conclude that in HeLa cells alteration of mRNA levels occurs in most of the H1 genes and controls at all doses tested, including the lowest dose that we have seen previously that did not alter cell physiology. In general, the higher increase in the messenger occurs at 50 mM and 75 mM. These results are supported by the analysis of m6A inhibition by dot blot (Figure 8.) where we can observe that the levels of m6A are somehow restored at 100mM of *cycloleucine*.

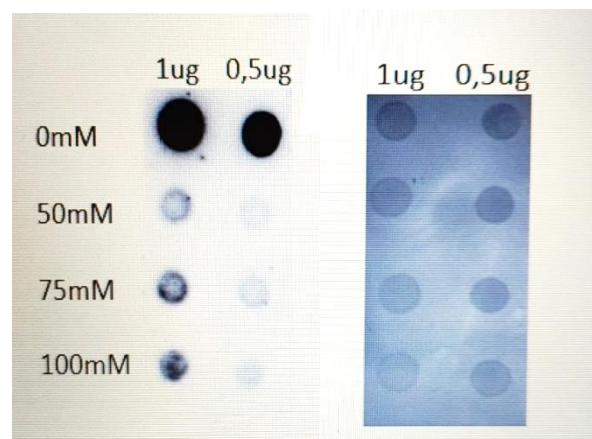


Figure 8. Analysis of m6A content in HeLa at different doses of *cycloleucine*. The right panel shows the dot blot using anti-m6A antibody. The concentrations correspond to the dose of the inhibitor. The left panel shows the RNA loading control stained with methylene blue.

We also analysed mRNA levels after *cycloleucine* treatment in other cell line HEK293T (Figure 9). In the case of H1 an increase of mRNA levels was found

in H1.0 and H1.1 at low doses of the inhibitor. The rest of H1 subtypes remained unaltered at low doses. At 100 mM all H1 subtypes were decreased, except for H1.1 and H1X that show a slight increase. In this cell line BRCA1, MYC and METTL14 show behaviour similar to H1.0, while the rest of the genes analysed showed variable effects.

The treatment with the inhibitor had less effect in HEK293T cell line as the max fold change was 3.5 compared with 9.4 in HeLa, suggesting that the m6A could have less impact in this cell line.

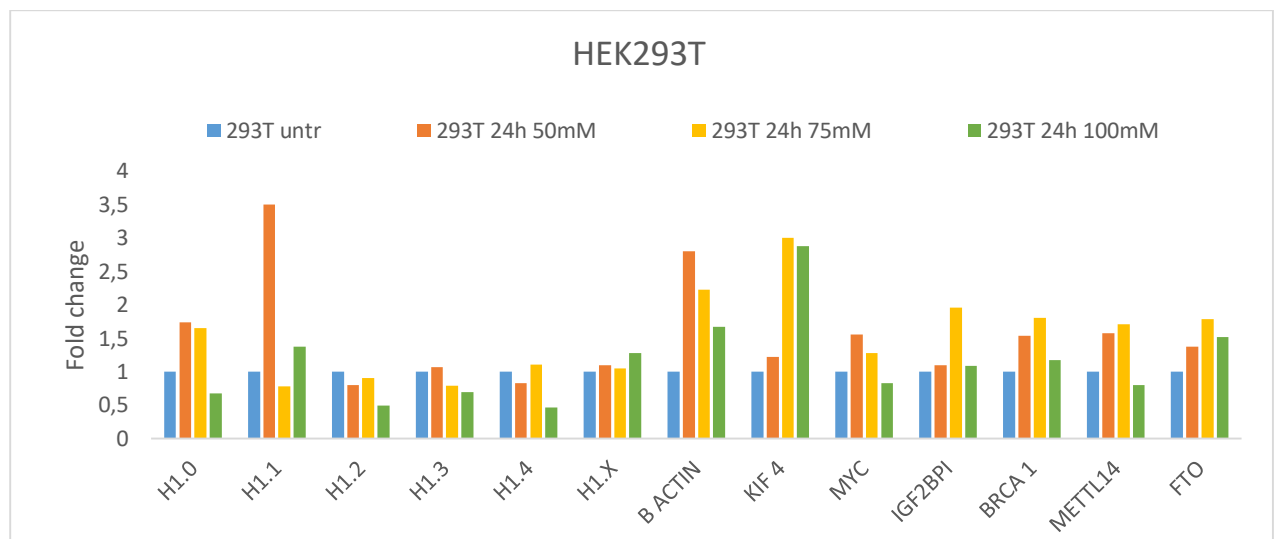


Figure 9. HEK293T effect of METTL3 on mRNA levels

In general, these results suggest that the role of m6A in the regulation of H1 is differential among subtypes and cell-type specific.

4.3. Effect of METTL3 inhibition on H1 protein levels

Moreover, it was analysed if the changes in the mRNA levels of H1 subtypes upon METTL3 inhibition also affected the protein levels. HeLa cells were treated with 100mM dose of inhibitor for 24h. Figure 10. reveals that, when treated with *cycloleucine* there is an increase in all H1s except in H1.2 and H1.4 which are decreased.

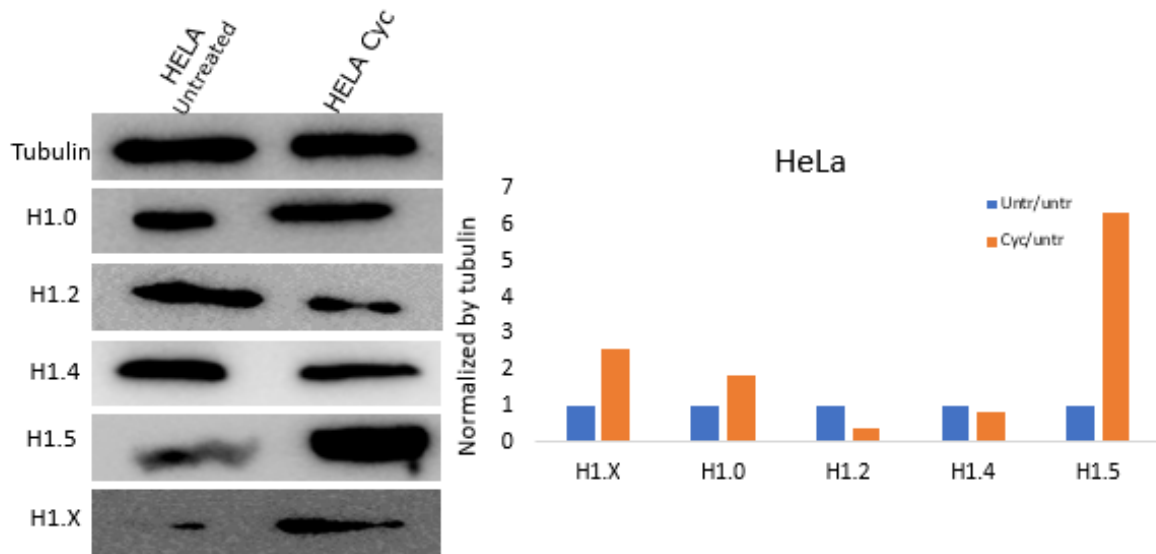


Figure 10. Western blot analysis of HeLa treated with *cycloleucine* (on the left) and WB quantification (on the right).

The direction of the change in the protein levels (increase or decrease) agrees with the changes observed in the mRNA, except for H1.4. In this subtype, there is an accumulation at mRNA level and a decrease at the protein level. Considering the magnitude of the change, there is no correspondence between mRNA and protein levels. In general, the effects are different among subtypes supporting the idea that m6A plays a subtype specific role in H1 regulation.

4.4. Proteasome inhibition

To study the translational regulation of histone H1 we analysed the role of the proteasome in the degradation of H1 subtypes. Previous experiments in T47D have shown that H1 subtypes accumulate following proteasome inhibition, indicating its involvement in H1 degradation. Furthermore, the group has also shown that H1 can be degraded in vitro by the 20S proteasome suggesting that as histone H1 has two domains that are intrinsically disordered, this mechanism could function in the case of H1 degradation. Previous group results also indicate that the degradation by

the 20S proteasome is dependent on the c-terminal domain. Experimentally we set out to determine if H1 subtypes were accumulated upon proteasome inhibition in other cell lines, HeLa and HEK293T, as a prove of its involvement in the control of protein levels.

We used two different inhibitors MG132 and bortezomib and checked the accumulation by western blot. The doses of drugs and treatment times are indicated in the methods section.

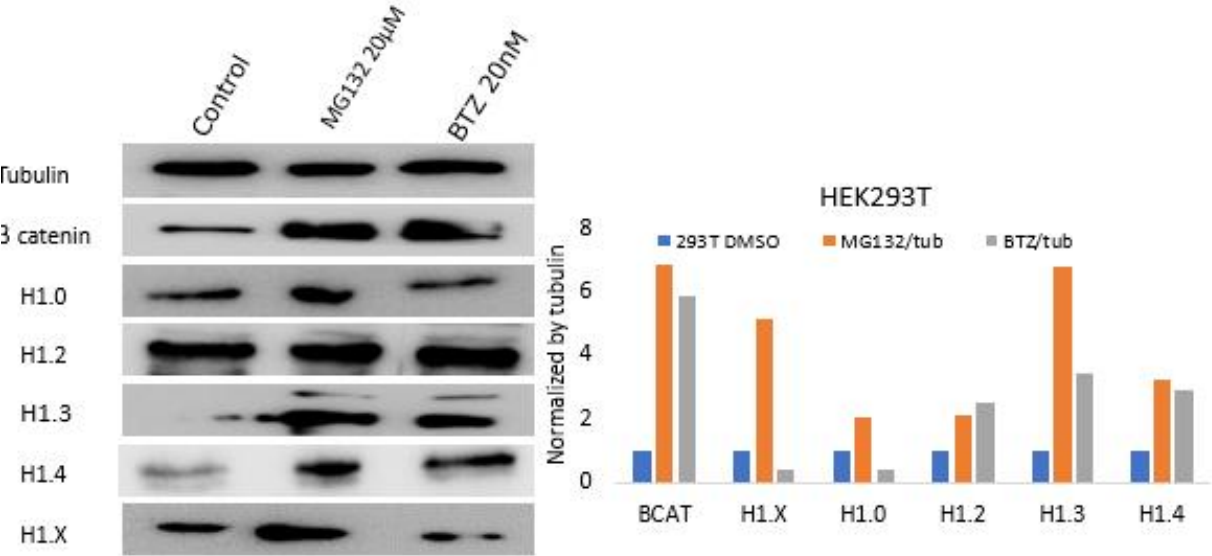


Figure 11. Accumulation of H1 subtypes by proteasome inhibition in HEK293T (on the left), and western blot quantification (on the right). Two inhibitors were used MG132 at 20 μ M dose, and bortezomib at 20nM dose.

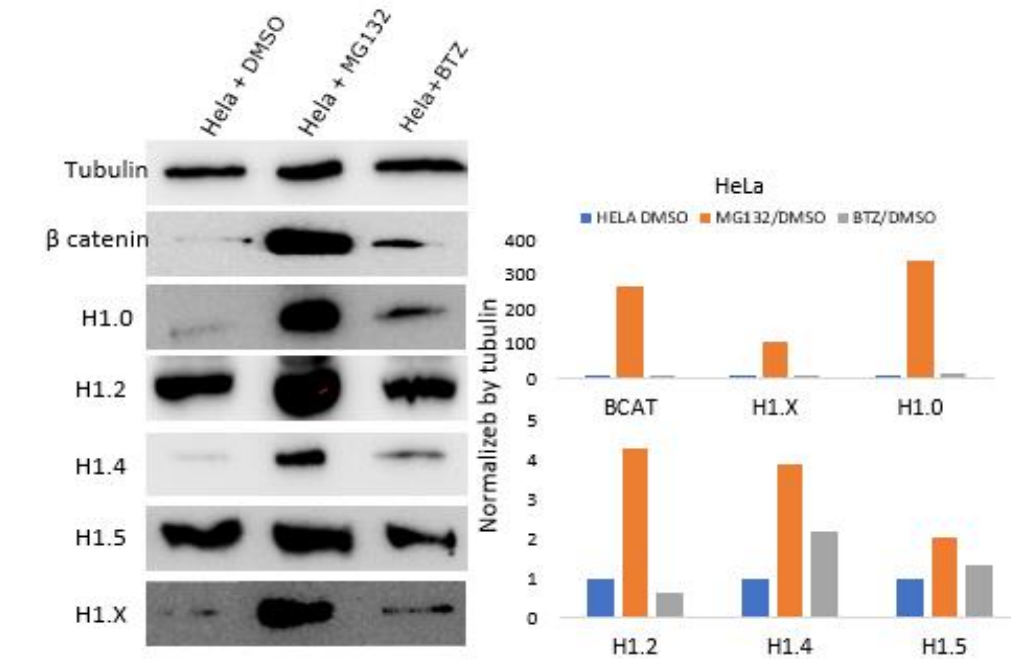


Figure 12. Accumulation of H1 subtypes by proteasome inhibition in HeLa cells (on the left), and western blot quantification (on the right). Two inhibitors were used MG132 at 20 μ M dose, and bortezomib at 20nM dose.

We used the same experimental procedure in HeLa like in HEK293T and the results were (figure 11 and figure 12):

As in the previous cell line, all H1 subtypes accumulate upon MG132 treatment. All H1 subtypes, except H1.2, accumulated upon bortezomib treatment, although the accumulation was lower than the observed in MG132, even in the positive control, suggesting some resistance to the dose of bortezomib that was used.

Summarizing the results in both cell lines we found the same behavior in MG132, but the accumulation upon bortezomib treatment was different among cell lines in the magnitude of the accumulation and in the subtypes that were affected, suggesting that the regulation of the protein levels of H1 subtypes is cell-line specific.

5. Discussion

The main objective of this study is to understand Histone H1 subtypes regulation. As a part of this global aim there are two projects. The first one is regarding to the role of m6A in Histone H1 regulation. To establish this, *cycloleucine* was used as inhibitor of METTL3, enzyme that incorporates m6A in the mRNA. *Cycloleucine* is non-metabolisable amino acid formed through the cyclization of leucine. Likewise, it is a specific and reversible inhibitor of nucleic acid methylation and it is generally used in biochemical experiments (18). This inhibitor was used in two different cell lines HEK293T and HeLa. By inhibiting enzyme METTL3, it was observed that by increasing the dose of inhibitor there is a greater effect on the growth of the cells. That effect is manifested in both HEK293T and HeLa cell line. As histone H1 transcript levels change during cell cycle, parallel to growth kinetics these changes in cell cycle were analyzed by Flow cytometry. It was noticed that the addition of *cycloleucine* does not significantly affect the percentage of HeLa cells in each phase at 24h. There is a consistent alteration at higher dose of 100mM at 48h where an accumulation of cells in G2M phase occurs, so for the rest of the analysis the treatment was performed during 24h. The cell cycle has a role in cancer progression through regulation of cancer cell division. It was reported that silencing METTL14 or ALKBH5 inhibits cell cycle by arresting in the G1-S phase in breast cancer (22).

To determine effect of METTL3 inhibition on mRNA levels as controls were used cell-cycle related genes such as MYC and BRCA1. In addition, were used some genes associated with m6A metabolism such as METTL14, IGF2BP1, and FTO, and a housekeeping gene β -actin. Researchers have reported that FTO can regulate the development of many cancers. Upregulated FTO can act as an oncogene in lung squamous cell carcinoma as well as be an indicator of poor prognosis. FTO knockout promotes apoptosis and inhibits cell proliferation, whereas FTO knockdown showed to significantly inhibit MZF1 levels, therefore inhibit lung cancer cell viability and cell invasion. These were important findings for epigenetic changes that

provided possible therapeutic targets for patients with LUSC (22). In HeLa cells, it has been reported that knockdown of METTL14 decreases the m6A level more significantly than silencing METTL3 (23). For that reason, we used an inhibitor in this study because knockdown of METTL3 did not show promising results. METTL14 mainly functions as an RNA-binding scaffold, which recognizes substrate RNA and stabilizes the combination between METTL3 and SAM (23).

Our results show that in HeLa cells the subtypes, H1.2, H1.0 and H1.4, are the ones that present the most significant alterations in all doses of inhibitor. This is consistent with previous MeRIP data where these subtypes are shown to be highly methylated (unpublished group results). In other cell line, HEK293T, (Figure 8.) an increase of mRNA levels was found in H1.0 and H1.1 at low doses of the inhibitor. The rest of H1 subtypes remained unaltered at low doses. Further, the treatment with the inhibitor had less effect in HEK293T cell line as the max fold change was 3,5 compared with 9,4 in HeLa. In the case of HeLa, where we can correlate mRNA levels, and protein levels we can hypothesize about the role of m6A in the regulation of H1s. It seems that in the case of H1.0, H1.5 and H1X m6A plays a role in mRNA degradation because there is an increase in the transcript and protein levels following METTL3 inhibition. In contrast, for H1.2, the role of m6A seems to be associated with mRNA stabilization as suggested by the decrease in mRNA and protein levels. This idea is supported also by a RIP-MS analysis performed with an H1.2 specific probe made in the group (unpublished results). In this experiment the m6A readers found enriched in the H1.2 transcript are associated with mRNA stabilization and storage. Finally, in the case of H1.4 the mRNA levels increase after *cycloleucine* treatment, but the protein levels are decreased suggesting that m6A is associated with the increase of translation efficiency. Further experiments need to be performed to confirm or reject these hypotheses.

In general, we could say that these results suggest that the role of m6A in the regulation of histone H1 is differential among subtypes and is cell-type specific.

On the other hand, my second project was to study the role of the proteasome in degradation of H1 subtypes. To determine whether the proteasome is involved in the degradation of histone H1 subtypes in two different cell lines HeLa and HEK293T were used two inhibitors, MG132 and Bortezomib and analysed the protein levels by western blot. Tubulin was used as a loading control and β -catenin as a positive control because it is widely known that is targeted for proteasomal degradation (19).

Western blot analysis showed that all H1 subtypes accumulate upon MG132 treatment in both cell lines. Likewise, in HeLa all H1 subtypes, except H1.2, accumulated upon bortezomib treatment, although the accumulation was lower than the one observed in MG132, even in the positive control, suggesting some resistance to the dose of bortezomib that was used. In HEK293T cells treated with bortezomib, only the replication-dependent subtypes are accumulated. H1.3 subtype was the only one with higher accumulation in both inhibitors. The differences between the results with both inhibitors could be explained by their mechanism of action. The peptide MG132 is a proteasome inhibitor that binds to the active site of the β -subunits, inhibits 20S proteasomal activity, therefore, blocks the proteolytic activity of the 26S proteasome complex. MG132 induces cell cycle arrest and consequently inhibits growth of tumoral cells as well as trigger apoptosis (24). Bortezomib can block chymotrypsin-like activity of the proteasome by binding and forming a complex with the active site of threonine hydroxyl group in the β 5-subunit. Alongside MG132, bortezomib is inhibiting proteasome activity by reversibly binding to the chymotrypsin-like (CT-L) subunit of the 26S proteasome complex. This results in its inhibition, and it prevents the degradation of various pro-apoptotic factors. The accumulation eventually activates the programmed cell death via caspase-mediated pathways (25). MG132 can inhibit the growth of

tumor cells by inducing the cell cycle arrest or triggering apoptosis. There are few reported pathways which induce apoptosis. For instance, through formation of reactive oxygen species (ROS), through cooperation with APO2L, tumor necrosis factor (TNF)-related apoptosis inducing ligand (trail) or through p53-independent pathway (26). MG132 and bortezomib have similar mechanisms of proteasome inhibition but there is a slight difference. For instance, besides proteasome inhibition MG132 can affect many different pathways, including other proteolytic enzymes, compared to bortezomib which binds directly to the CT-L domain and inhibits proteasome degradation (25). The mechanism of action of the different inhibitors can also contribute to the differences between subtypes, together with the differences in protein stability.

Other factors that could contribute to the differences in the results between the inhibitors are the resistance to drug and the dose used. Resistance to effect of bortezomib that was reported in studies so far are like what has been previously reported with bortezomib adapted hematologic-derived tumor cell lines. Mechanism of resistance includes increase in proteasome activity, mutations in the $\beta 5$ subunit and genetic alterations in stress response and cell survival pathways. This suggest that resistance to this dipeptide boronated proteasome inhibitor is independent of tumor cell lineage (18). The stated independence may be the reason for the obtained results which indicate low accumulation of H1s in HeLa and furthermore, low accumulation of the positive control β catenin.

Recent studies have found that proteasome inhibitors can affect protein turnover but at much higher concentrations than those achieved clinically, indicating that some of the effects of proteasome inhibitors are mediated by other mechanisms (18). Several studies have reported that protein turnover of critical cellular proteins is altered by the treatment of cells with proteasome inhibitors. For instance, the doses of bortezomib required to alter protein levels are usually much higher than the doses required for cytotoxicity. For example, one study tested 5 μM of bortezomib for 1 h and

found reduced degradation of I κ B α in response to tumor necrosis factor. Other studies used micromolar levels of bortezomib and treatment times of 6 hours or more to demonstrate different levels of protein (18). Considering that different doses of inhibitors were used, there is a possibility that this affects achieved results. Furthermore, the possibility of the proteasome degradation could be associated with some specific PTM accounting for the difference among subtypes. Posttranslational modifications such as phosphorylation, glycosylation, N-acetylation, poly-ADP ribosylation, ubiquitination have also been reported for numerous proteasome subunit proteins. Most of these modifications have been found to make alterations in proteasome activity, stability, assembly, even sensitivity to proteasome inhibitors (18) and could be associated with some of the differences observed among cell lines.

Summarizing the results in both cell lines, we found the same behavior in MG132, but the accumulation upon bortezomib treatment was different among cell lines in the magnitude of the accumulation and in the subtypes that were affected, suggesting that the regulation of the protein levels of H1 subtypes is cell-line specific. We also found differences among the H1 subtypes expressed within the same cell, suggesting that the regulation at the protein levels is also subtype-specific.

6. Conclusions

Regarding the role of m6A in H1 regulation we can conclude that:

- 1) Treatment with *cycloleucine* affects cell growth in both HeLa and HEK293T in a dose-dependent manner.
- 2) Treatment with *cycloleucine* for 48h cause an accumulation of cells in G2/M, while the treatment for 24 has some effects in HEK293T and virtually no effects in HeLa.
- 3) Upon *cycloleucine* treatment, mRNA levels increase in the H1 genes and controls at all doses, except H1.2 that mostly decreases.
- 4) The protein levels of H1.0, H1.3, H1.5 and H1X increase upon treatment with *cycloleucine*, while that of H1.2 and H1.4 decrease.
- 5) The effects of the *cycloleucine* treatment are different among H1 subtypes and the changes at protein level not always reflect the changes in mRNA levels.

The general conclusion is that m6A plays a subtype specific role in H1 regulation.

Summarizing the results for proteasomal degradation,

- 1) In both cell lines (HeLa and HEK293T) there is accumulation of all H1 subtypes upon treatment with MG132.
- 2) The accumulation upon bortezomib treatment is different among cell lines in the magnitude of the accumulation and in the subtypes that were affected.

These results suggested that the degradation of H1 subtypes by the proteasome is subtype- and cell-type specific.

7. Literature

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