UNIVERSITAT POMPEU FABRA

FACULTAD DE CIÈNCIES DE LA SALUD I DE LA VIDA

TESIS DOCTORAL

Role of linker histone H1 variants in cell proliferation, chromatin structure and gene expression in breast cancer cells.

Mónica Sancho Medina

Mayo 2008

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Trabajo presentado por Mónica Sancho Medina para optar al grado de Doctora. Dirección a cargo de los doctores Albert Jordan y Miguel Beato en el Centro de Regulación Genómica (CRG) en Barcelona.

Mayo 2008

Dr. Albert Jordan Vallès
Staff Scientist/Subgroup Leader
Centre de Regulació Genòmica

Dr. Miguel Beato del Rosal
Director/Senior Group Leader
Centre de Regulació Genòmica

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SUMMARY

At least eleven histone H1 variants exist in mammalian somatic cells that bind to the linker DNA and stabilize the nucleosome particle contributing to higher order chromatin compaction. In addition of playing a structural role, H1 seems to be involved in the activation and repression of gene expression. It is not well known whether the different variants have specific roles or regulate specific promoters. We have explored this by inducible shRNA-mediated knock-down of each of the H1 variants in a human breast cancer cell line. Rapid inhibition of each H1 variant was not compensated by changes of expression of other variants. A different, reduced subset of genes is altered in each H1 knock-down. Interestingly, H1.2 depletion represses expression of a number of cell cycle genes. This is concomitant with a G1 arrest phenotype observed in this cell line. In addition, H1.2 depletion caused decreased global nucleosome spacing. These effects are specific of H1.2 depletion as they are not complemented by overexpression of other variants and they do not occur in knock-downs for the other variants. Moreover, H1.4 depletion caused cell death in T47D, being the first report of the essentiality of an H1 variant for survival in a human cell type.

In addition to this, we have also investigated specificities of H1 subtypes location in particular promoters of interest in our laboratory, as well as specific interactions with other factors by generating HA-tagged H1 variant expressing cell lines.

Al menos once variantes de la histona H1 han sido identificadas en mamíferos, todas ellas se unen al ADN entre nucleosomas contribuyendo así a la estabilización de la partícula nucleosómica y a la compactación de la cromatina en estructuras de alto orden. Además de jugar un papel estructural, H1 parece estar implicada en la activación y represión de la expresión génica. Se comprende escasamente si las diferentes variantes de H1 tienen funciones específicas o regulan promotores específicos. Con el objetivo de investigar esta hipótesis se han generado líneas celulares que inhiben de forma inducible mediante la tecnología de ARN interferente la expresión de cada una de las variantes de forma específica. La inhibición de cada una de las variantes no es compensada por cambios en la expresión del resto de subtipos. Distintos grupos de genes resultan alterados con la depleción de cada una de las variantes de H1. Sorprendentemente, la inhibición de H1.2 reprime la expresión de una serie de genes de ciclo celular, correlacionando con un fenotipo de arresto celular en fase G1 observado en esta línea. Además, la deleción de H1.2 causa una disminución global del espaciamiento entre nucleosomas. Todos estos efectos parecen ser específicos para la falta de H1.2 ya que no son complementados por la sobreexpresión de otras variantes. Por otro lado, la inhibición de H1.4 causa muerte celular en T47D. Ésta es la primera vez que se describe que una variante de H1 es esencial para la supervivencia de una línea celular humana.

En un segundo plano, se han construido líneas celulares con expresión de las variantes de H1 fusionadas al péptido HA, con el objetivo de estudiar la especificidad de su localización en promotores de interés para el grupo, así como interacciones específicas con otros factores celulares.

INTRODUCTION

1. Chromatin structure and dynamics.

1.1- The nucleosome particle.

The DNA is a right-handed double helix structure formed by two anti-parallel strands. Each strand is the succession of four nucleotides: Adenine (A), Thymine (T), Guanine (G) and Cytosine (C), bond by phosphodiester bonds. Adenine can pair only with Thymine and Cytosine with Guanine. The two strands are held together by hydrogen bonds between base pairs. Approximately, 146 base pairs of doubled-stranded DNA are wrapped around an octamer containing two each of highly conserved core histone proteins: H2A, H2B, H3 and H4. This structure received the name of nucleosome (Figure 1) and is the fundamental repetitive unit of the chromatin (Kornberg and Thomas 1974; van Holde 1988; Wolffe 1998).

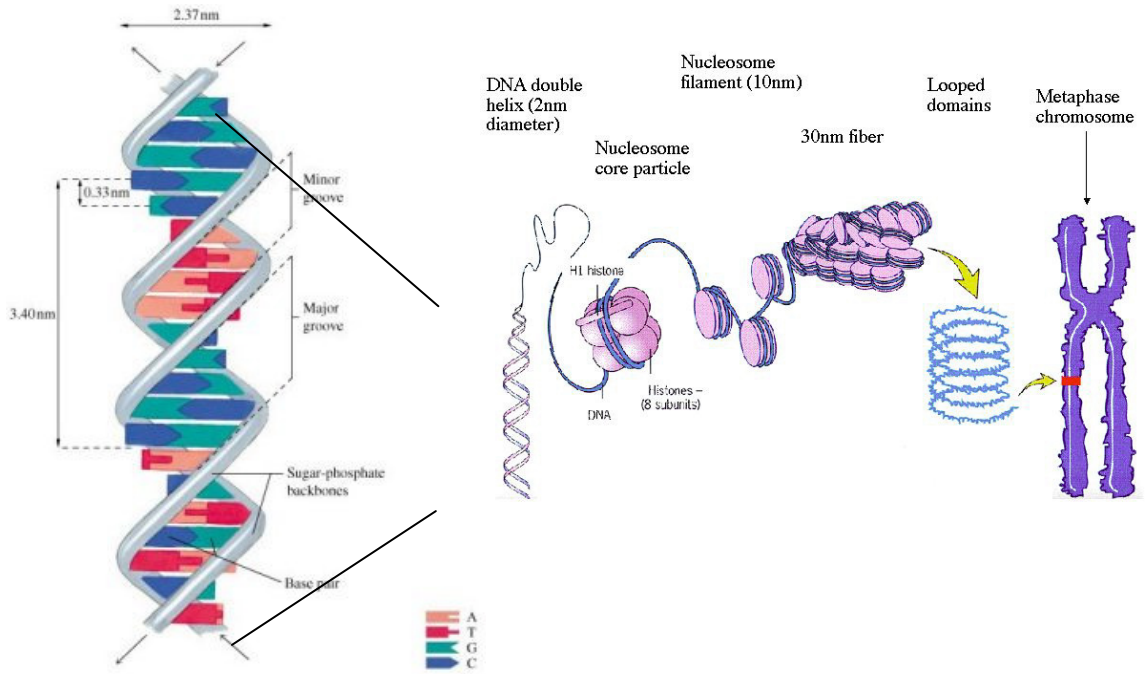


Figure 1. Schematic representation of the different levels of DNA compaction, from the double helix to the chromosome.

Chromatin also contains a fifth histone, the linker histone or usually called H1, which binds to the linker DNA (20 - 30bp) between nucleosomes, stabilizing them. Although its precise location in the nucleosome remains to be determined, it is clear that histone H1 sits outside of the core particle to form the chromatosome and plays a crucial role in folding the chromatin into more compact structures such as the high-order 30nm fiber (Figure 1).

Such 30nm fibers, are then further condensed *in vivo* to form 100-400 nm thick interphase fibers or the more highly compacted metaphase chromosome structures (Ramakrishnan 1997; Widom 1998; Belmont 1999, Zlatanova et al. 1999; Woodcook and Dimitrov 2001). Since DNA is supercoiled around the octamer in the nucleosome, and the nucleosomes can be organized into 30 nm filament, the association of histones with DNA results in considerable compaction and allows DNA that is over a meter long ($3*10^9$bp) to be organized in a cell nucleus that is only microns (10 um) in diameter.

However, the packing of DNA into chromatin restricts the access to DNA of many regulatory proteins necessary for the maintaining of biological functions such as gene transcription, DNA replication or DNA repair. Consequently, such structures must be dynamic and capable of regulated unfolding-folding transitions. There are several mechanisms that collaborate together to control the repressive nature of chromatin, allowing access to nucleosomal DNA: posttranslational modification of histone tails by specific enzymes, replacement of specific histone variants and alteration of nucleosomal structure by ATP dependent chromatin remodelling complexes.

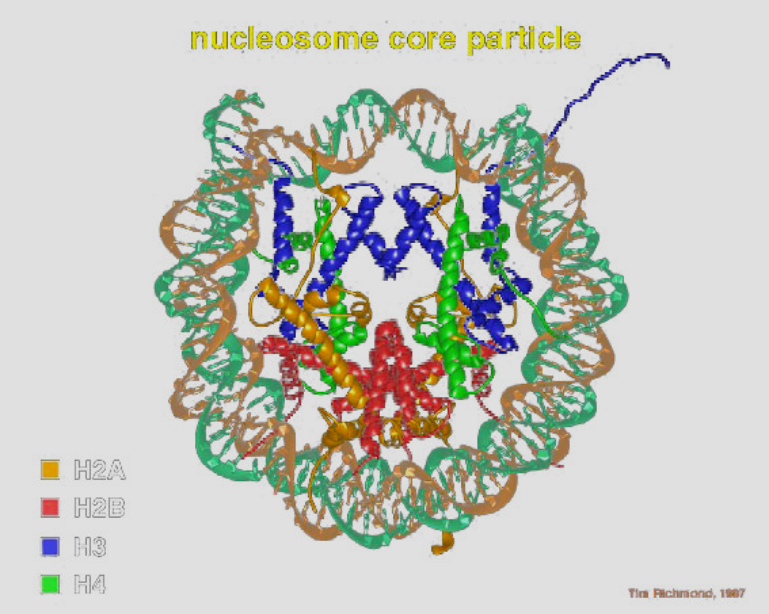


Figure 2. Structural model for the nucleosome core particle.

1.2- The core histones.

Core histones are small basic proteins remarkably conserved in length and amino acid sequence through evolution. Each of the core histones has a related globular domain that mediates histone–histone interactions within the octamer, and that organizes the two wraps of nucleosomal DNA. Each histone also harbours an amino terminal 20-35 residue segment that is rich in basic amino acids (Lys and Arg) and extends from the surface of the nucleosome; histone H2A is unique in having an additional ~37 amino acid carboxy-terminal domain that protrudes from the nucleosome. These histone 'tails' do not contribute significantly to the structure of individual nucleosomes nor to their stability, but they do play an essential role in controlling the folding of nucleosomal arrays into higher order structures. Although the highly basic histone tails are generally viewed as DNA-binding modules, their essential roles in tail-mediated chromatin folding also involve inter-nucleosomal histone-histone interactions (Peterson and Laniel 2004).

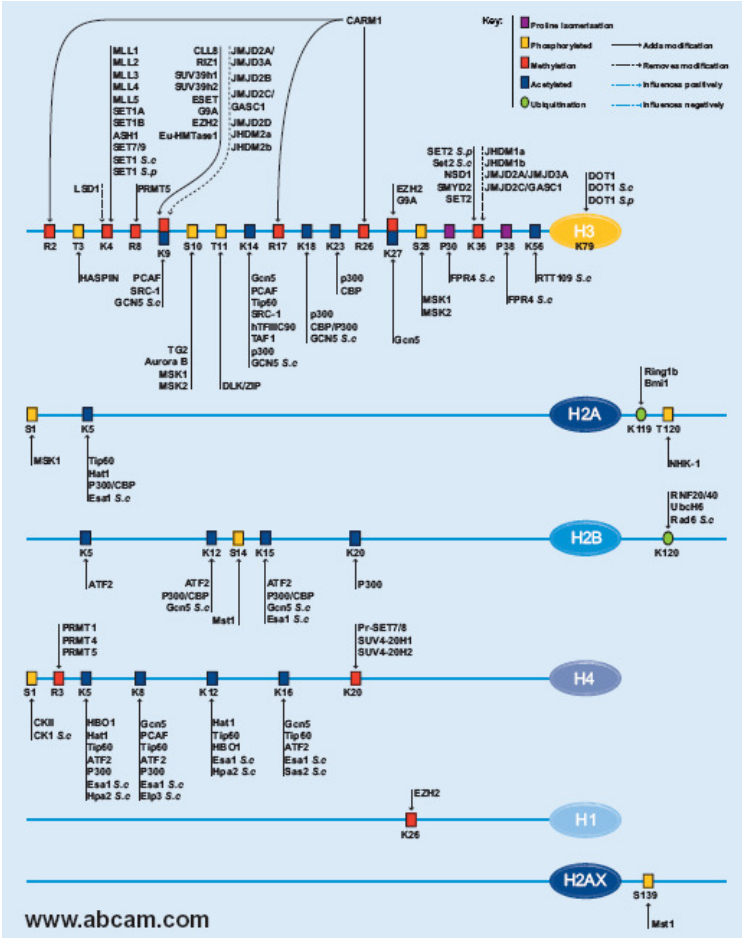


Figure 3. Map of histone modifications and enzymes involved in their regulation (image from Abcam).

Histones are subject to posttranslational modifications, including acetylation and methylation of lysines (K) and arginines (R), phosphorylation of serines (S) and threonines (T), ubiquitylation and sumoylation of lysines, as well as ribosylation (Figure 3).

Recent studies have shown that site-specific combinations of histone modifications correlate well with particular biological functions (Table 1). For instance, tri-methylation of H3 K9 and the lack of H3 and H4 acetylation correlate with transcriptional repression in higher eukaryotes. Moreover, phosphorylation of histone H2A (at S1 and T119) and H3 (at T3, S10 and S28) appear to be hallmarks of condensed mitotic chromatin. These and other observations have led to the idea of a histone modification 'code' which might be read by various cellular machineries, that is, a particular combination of histone marks will dictate the same biological function (Peterson and Laniel 2004). However, there have been described clear exceptions, a particular modification can have different or even opposite biological consequences. Thus, rather than a histone code there are instead clear patterns of histone marks that can be differentially interpreted by cellular factors, depending on the gene being studied and the cellular context.

These specific histone modifications control the binding of non histone proteins to the chromatin fiber that elicit the function associated with a particular histone mark. For example, recruitment of ATP-dependent remodelling enzymes such as SWI/SNF complexes, Polycomb repressive complexes, transcription factors and so on. In addition to this, there have been identified several variants of some core histones (Table 2). In most cases, how histone variants alter nucleosome structure or change the folding properties of chromatin is not known. Thus, incorporation of histone variants into chromatin fibers might enhance chromosome dynamics by creating domains of chromatin with novel properties (Peterson and Laniel 2004).

Table 1. Summary of the main modifications identified in core histones and their biological functions associated (reviewed by Peterson and Laniel 2004).

Modification	Histone	Site	Enzyme	Possible function
Acetylation	H2A	K4 (*S. cerevisiae*)	Esa1	Transcriptional activation
		K5 (mammals)	Tip60	Transcriptional activation
			p300/CBP	Transcriptional activation
		K7 (*S. cerevisiae*)	Hat1	?
			Esa1	Transcriptional activation
	H2B	K5	ATF2	Transcriptional activation
		K11 (*S. cerevisiae*)	Gcn5	Transcriptional activation
		K12 (mammals)	p300/CBP	Transcriptional activation
			ATF2	Transcriptional activation
		K16 (*S. cerevisiae*)	Gcn5	Transcriptional activation
			Esa1	
		K15 (mammals)	p300/CBP	
			ATF2	Transcriptional activation
		K20	p300	Transcriptional activation
	H3	K4	Esa1	Transcriptional activation
			Hpa2	?
		K9	?	Histone deposition
			Gcn5	Transcriptional activation
			SRC-1	Transcriptional activation
		K14	Gcn5, PCAF	Transcriptional activation
			Esa1, Tip60	Transcriptional activation
				DNA repair
			SRC-1	Transcriptional activation
			Elp3	Transcription elongation
			Hpa2	?
			hTFIIIC90	RNA polymerase III transcription
			TAF1	RNA polymerase II transcription
			Sas2	Euchromatin?
			Sas3	Transcriptional activation/elongation?
			p300	Transcriptional activation
		K18	Gcn5 (SAGA/STAGA complex)	Transcriptional activation
				DNA repair
			p300, CBP	DNA replication
				Transcriptional activation
		K23	Gcn5 (SAGA/STAGA complex)	Transcriptional activation
			Sas3	DNA repair
			p300, CBP	Transcriptional activation/elongation?
				Transcriptional activation
		K27	Gcn5	Transcriptional activation
	H4	K5	Hat1	Histone deposition
			Esa1, Tip60	Transcriptional activation
				DNA repair
			ATF2	Transcriptional activation
			Hpa2	?
			p300	Transcriptional activation
		K8	Gcn5, PCAF	Transcriptional activation
			Esa1, Tip60	Transcriptional activation
				DNA repair
			ATF2	Transcriptional activation
			Elp3	Transcription elongation
			p300	Transcriptional activation
		K12	Hat1	Histone deposition
				Telomeric silencing
			Esa1, Tip60	Transcriptional activation
				DNA repair

Modification	Histone	Site	Enzyme	Possible Function
		K12	Hpa2	?
		K16	Gcn5	Transcriptional activation
			MOF (*D. melanogaster*)	Transcriptional activation
				Transcriptional activation
			Esa1 (yeast), Tip60 (mammals)	DNA repair
			ATF2	Transcriptional activation
			Sas2	Euchromatin
Methylation	H3	K4	Set1 (yeast)	Permissive euchromatin (di-Me)
			Set9 (vertebrates)	Active euchromatin (tri-Me)
				Transcriptional elongation/memory (tri-Me)
				Transcriptional activation
			MLL, Trx	Transcriptional activation
			Ash1 (*D. melanogaster*)	Transcriptional activation
		K9	Suv39h, Clr4	Transcriptional silencing (tri-Me)
				DNA methylation (tri-Me)
			G9a	Transcriptional repression
				Imprinting
			SETDB1	Transcriptional repression (tri-Me)
			Dim-5, Kryptonite	DNA methylation (tri-Me)
			Ash1 (*D. melanogaster*)	Transcriptional activation
		R17	CARM1	Transcriptional activation
		K27	Ezh2	Transcriptional silencing
				X inactivation (tri-Me)
		K36	Set2	Transcriptional elongation
				Transcriptional repression?
		K79	Dot1p	Euchromatin
				Transcriptional elongation / memory
	H4	R3	PRMT1	Transcriptional activation
		K20	PR-Set7	Transcriptional silencing (mono-Me)
			Suv4-20h	Heterochromatin (tri-Me)
			Ash1 (*D. melanogaster*)	Transcriptional activation
		K59	?	Transcriptional silencing?
Phosphorylation	H2A	S1	?	Mitosis
			?	Chromatin assembly?
			MSK1	Transcriptional repression
		T119	NHK-1	Mitosis
		S129 (S. cerevisiae)	Mec1	DNA repair
		S139 (mammalian H2AX)	ATR, ATM, DNA-PK	DNA repair
	H2B	S14 (vertebrates)	Mst1	Apoptosis
		S33 (D. melanogaster)	TAF1	Transcriptional activation
	H3	T3	?	Mitosis
		S10	Aurora-B kinase	Mitosis, meiosis
			MSK1, MSK2	Immediate-early activation
			Snf1	Transcriptional activation
		T11 (mammals)	Dlk/ZIP	Mitosis
		S28 (mammals)	Aurora-B kinase?	Mitosis
			MSK1, MSK2	Immediate-early activation
	H4	S1	?	Mitosis
Ubiquitylation	H2A	K119 (mammals)	HR6A,B?	Spermatogenesis
	H2B	K120 (mammals)	HR6A,B?	Meiosis
		K123 (S. cerevisiae)	Rad6	Transcriptional activation
				Euchromatin
	H3	?	?	Spermatogenesis
Sumoylation	H4	?	Ubc9	Transcriptional repression

Histone	Variant			Functional association
	Mammals	Yeast	*Drosophila*	
H3	H3.1	–	–	S-phase subtypes
	H3.2	–	–	S-phase subtypes
	H3.3	H3.3	H3.3	Transcriptionally active regions
	Cenp-A	Cse4	Cid	Centromeric nucleosomes
H2A	H2A.Z	Htz1	H2Av[a]	Different functions in various organisms: maintenance of pericentric and telomeric heterochromatin, transcriptional activation and viability
	H2A.X	H2A	H2Av[a]	Sex body in mammals, site of DNA double stranded breaks; condensation and silencing of male sex chromosome
	MacroH2A	–	–	Inactivation of X-chromosome, interferes with both transcription factor binding and SWI/SNF remodelling
	H2A.Bbd	–	–	Close spacing of nucleosomes

[a]*Drosophila melanogaster* has a single H2A variant, H2Av, in addition to the major H2A. H2Av is not only a member of H2A.Z family, it also contains an SQ motif similar to mammalian H2A.X. It is phosphorylated at Ser137 and hence it is a functional homologue of H2A.X.

Table 2. Functional diversity of histone H3 and H2A variants (reviewed by Bhargava 2005).

Histone variants, distinct patterns of posttranslational modifications of histones, and histone tail binding proteins all contribute to establishment of various ‘open’ or ‘closed’ chromatin domains that have specialized folding properties and biological functions.

1.3- Chromatin-remodelling complexes.

Nucleosomes positioned at promoters play an important role in the regulation of transcription (Truss et al. 1995). To allow access to the transcriptional apparatus, such nucleosomes have to be disrupted. Chromatin-modifying complexes can be categorized into two major classes depending on their mode of action:

a) enzymes that covalently modify the core histones.

b) enzymes that use the energy of ATP hydrolysis to disrupt chromatin structure.

The first class includes several groups of enzymes that are able to bind and modify core histones as it has been discussed previously. Some of them are summarize in table 2 according to the modification type. For instance, histone acetyltransferases (HATs) and histone deacetylases (HDACs) are important in the control of gene activation. Research has demonstrated that acetylation of the core histones tails neutralizes the positively charged lysine residues and decreases the affinity of the tails for DNA (Gross and Garrad 1988; Lee et al. 1993). It is thought that acetylation of the core histone tails opens the repressive chromatin structure and allows transcription factors access to the DNA (Bradbury 1998; Kornberg and Lorch 1999).

The second class of chromatin-remodelling complexes is the ATP-dependent chromatin-remodelling complexes that can be divided into a number of families depending on what type of ATPase subunit they possess. The two main families of multiprotein complexes involved in remodelling of chromatin during transcription are the SWI/SNF complex (Tsukiyama et al. 1994; Kwon et al. 1994) and the ISWI family. They differ in their components, mechanism and ATPase subunits but both families are able to move the DNA with respect to core histones. One of the most intriguing aspects of chromatin remodelling is the conflict between maintaining nucleosome organization while altering that organization on a local level for functional purposes (Hill 2001).

2. Linker histones.

2.1- General characteristics.

While involvement of core histones in the dynamics of chromatin has been extensively studied and characterized, the role of linker histone H1 remains still unclear. Histone H1 is a key component in chromatin compaction, bound near the entry and exit sites of the core particle stabilizes two full turns of DNA and facilitates the folding of the 30 nm fiber. The amount of H1 per nucleosome is variable, and the paradigm of one H1 per nucleosome is more the exception than the rule (Woodcock et al. 2006). The ratio H1/nucleosome can vary between cell types from 0.5 in ES to more than 1, specially in cells with repressed genomes (Bates et al. 1981; Woodcock 2006). Despite the binding of linker histone to the nucleosome is asymmetric, the precise location and orientation indicated by different studies still presents conflicting results. It is possible that H1 may not assume the same position or orientation on all nucleosomes. Distribution of H1 variants, modifications of core histones and the stability of individual nucleosomes would participate for determining the position or orientation of H1 on a nucleosome.

Most of the work on H1 was conducted on *in vitro* systems. H1-depleted and H1-containing chromatin were compared after dissociation of H1 from chromatin by ionic strength diminution in solution. Loss of H1 leads to decondensation of chromatin corresponding to a change in conformation from a zig-zag arrangement to a more open bead-on-a-string form. These experiments, together with studies showing a protection from nuclease digestion in H1 containing chromatin, provided a structural rationale for the role of H1 in sealing two complete turns of nucleosomal DNA. Moreover, H1-containing chromatin shows strong inhibition of nucleosome sliding (Hill 2001). Because of its positive charges, H1 plays a key role in chromatin compaction that is primarily an electrostatic event. *In vitro* studies suggested that H1 is required for formation and stabilization of higher order chromatin structure and acts as a general repressor of transcription.

Unicellular organisms such as *Tetrahymena thermophila*, *Saccharomyces cerevisiae* and *Ascobolus immerses* have only one H1 protein. Elimination of H1 in *Tetrahymena* (Shen et al. 1996) and in yeast (Hellauer et al. 2001) resulted in up- or down- regulation of specific genes while in *Ascobolus* resulted in abrupt stop of growth and increased accessibility of chromatin(Barra et al. 2000). In higher eukaryotes the number of H1 proteins increases. The worm *Caenorhabditis elegans* has eight H1 variants. Reduced expression of some of these subtypes also resulted in specific phenotypes such as severe defects in germline proliferation

and differentiation (Jedrusik et al. 2001). *Nicotiana tabacum* has two major (H1A and H1B) and 4 minor H1 variants. Aberrations in flower development and male sterility were observed when H1A and H1B content was reduced (Prymakowska-Bosak et al. 1999). In *Gallus gallus* there have been characterized six somatic subtypes in addition to H5 that is only expressed in highly differentiated cells (Takami et al. 1997). *Xenopus laevis* has five H1 variants, one of them oocyte –specific called B4. Reduction of H1 levels confirmed the repressive effect on specific genes (Bouvet et al. 1994). The majority of the data regarding the specific function of linker histones has come from studies in mice. Eleven subtypes have been identified and single knockout mice are viable with no obvious phenotype (Fan et al. 2001). Although remaining subtypes can compensate, H1 variants can indeed differentially modulate the level of transgene expression (Alami et al. 2003).

Linker histone H1 is more evolutionary diverse than other histones and, in fact, in humans we are talking about a heterogeneous family of proteins as in other higher eukaryotes (Doenecke et al. 1997; Parseghian and Hamkalo 2001). There have been described up to 11 variants, normally classified by the cell type in which they appear, most being found in somatic cells, while three (H1t, H1t2, and HILS1) are spermatogenic variants and one (H1oo) is oocyte-specific. The somatic subtypes can be further subdivided into replication-dependent and replication-independent variants. The first group (from H1.1 to H1.5) are expressed in S-phase, while the latter group contains H1.0, a replacement subtype found in growth arrested cells (Figure 4), and histone H1x, a recently described and still mysterious variant, that accumulates during G1 phase (Happel et al. 2005).

The five somatic subtypes have been referred to in the literature using many different nomenclature systems. In this report, we will use the numeric system. Table 3 gives a comparison of the different terms proposed in the literature.

Replication-dependent					
Human			Mammals		
Albig & Doenecke	Human official symbol	Ohe & Iwai	Mouse official symbol	Parsegian & Hamkalo	Seyedin & Kistler
H1.1	HIST1H1A		Histh1a	H1a	H1a
H1.5	HIST1H1B	H1a	Histh1b	H1s-3	H1b
H1.2	HIST1H1C	H1d	Histh1c	H1s-1	H1c
H1.3	HIST1H1D	H1c	Histh1d	H1s-2	H1d
H1.4	HIST1H1E	H1b	Histh1e	H1s-4	H1e
H1t	HIST1H1T		Histh1t		H1t
Replication-independent					
Human			Mammals		
Official symbol	Official full name	Alias	Official symbol	Alias	
H1F0	H1 member 0	H1.0	H1f0	H1(0)	
H1FNT	H1 testis-specific	H1T2	H1fnt	H1t2	
H1F00	H1 oocyte-specific	H1oo	H1foo	H1oo	
H1FX	H1 member X	H1x	H1fx	H1X	

Table 3. Overview of the most common nomenclatures of linker histone subtypes.

Every variant is codified by one intronless gene. Transcripts lack polyA tails but instead contain a palindromic termination element. H1 variant genes (Figure 4) are forming a large histone cluster in chromosome 6 except for H1.0 that is in chromosome 22 (Albig et al. 1993), H1x (Happel et al. 2005) and H1oo in chromosome 3, H1t2 in chr 12 and HILS1 in chr 17.

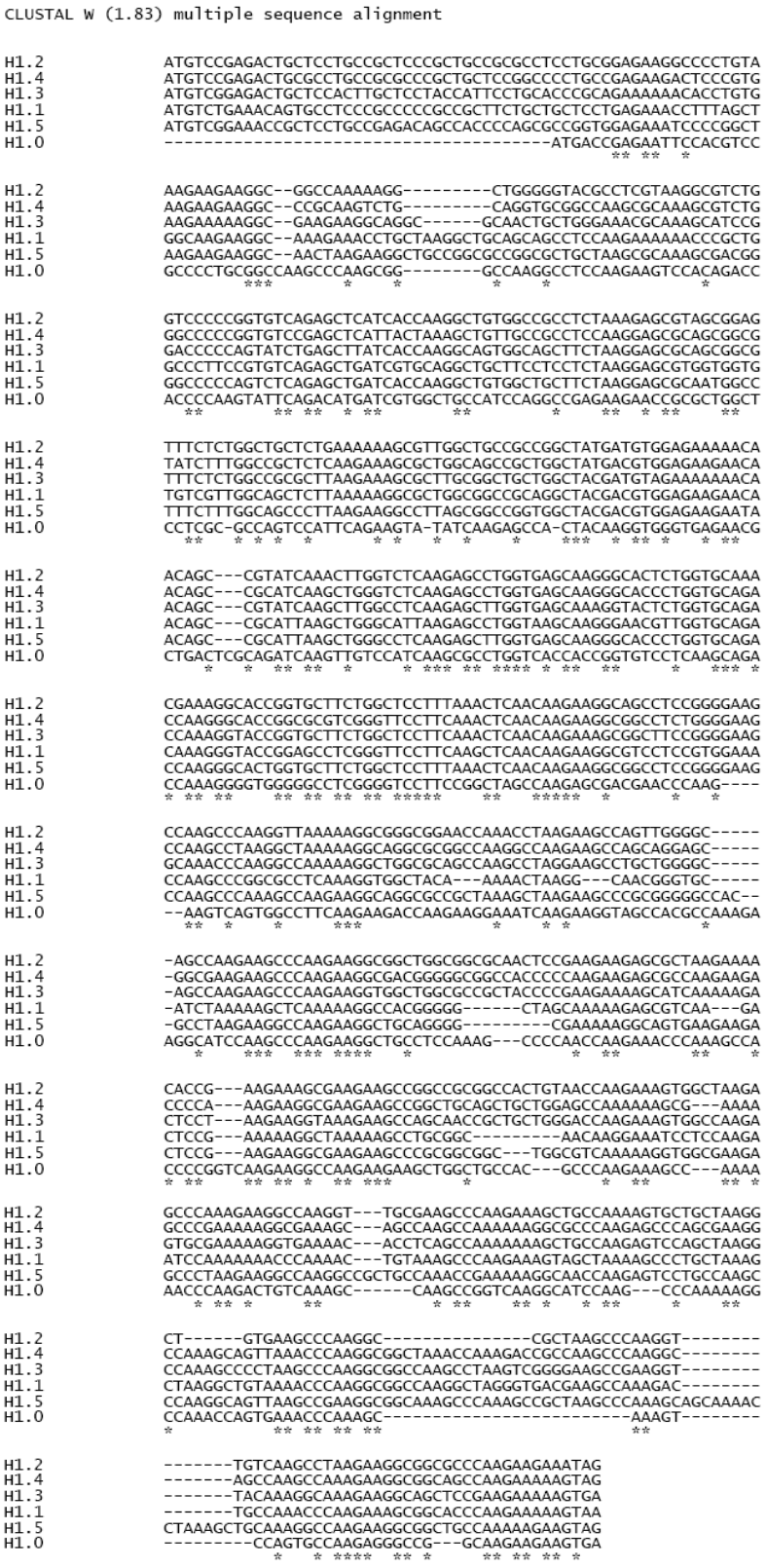


Figure 4. Alignment of human H1 somatic genes.

At the protein level, all of them share a common structural fold (Figure 5):

- a highly conserved central globular domain of approximately 80 aa long, responsible for DNA binding. It belongs to the “winged helix” family of DNA-binding proteins (Cerf et al. 1994).

- a more variable extended N- and C- terminal tails that function to modulate protein interaction with DNA. Both are enriched in basic residues, especially lysines. These tails are believed to be inherently flexible and largely unstructured in solution (Ramakrishnan 1997).

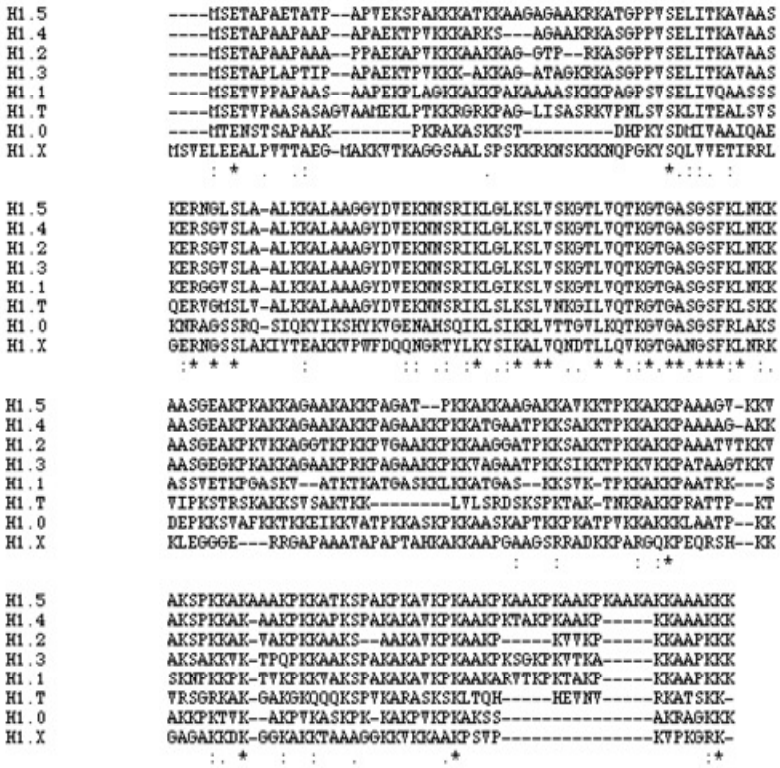


Figure 5. Alignment of the amino acid sequence of human H1 variants.

The contribution of particular variants to chromatin processes is currently an open question. This heterogeneity has been conserved among species suggesting that these individual subtypes may have unique properties in the cell (Brown 2001).

Historically, H1 was considered as a structural component, constantly associated to nucleosomes. Recent studies of fluorescence recovery after photobleaching (FRAP) of mouse cells expressing H1.0-GFP or H1c-GFP proteins showed that H1 is highly dynamic protein, continuously exchanging between chromatin sites (Misteli et al. 2000). H1 subtypes showed a broad range of recovery times due to differences in their binding affinity. The recovery time depends on the length of the C-terminal tail, as well as on the density of positively charged residues. Human H1.1 and H1.2 have the shortest tail and the most rapid recovery time, whereas H1.4 and H1.5 with longer C-terminal domains and more than two CDK phosphorylation motifs, show longer residence times. Both, H1.0 and H1.3, have intermediate properties and the highest content in positive residues (Th'ng et al. 2005).

Importantly, additional mechanisms are also participating for the binding affinity of H1 to chromatin. For instance, specific factors can promote the binding of H1 to unique location, competitors such as HMG proteins can weaken the interaction of H1 with chromatin, and post-translational modifications of core histones such as acetylation, can alter the binding affinities of H1 (Catez et al. 2006).

2.2- Particularities of histone H1 variants.

H1.1 (H1a)

Transcriptional evidence demonstrates that H1.1 is restricted to thymus, testis, spleen, lymphocytic and neuronal cells (Franke at al 1998; Rasheed et al. 1989). This subtype rapidly declines in amount as cells became quiescent, differentiated, or both (Lennox and Cohen 1983). In terms of post-translational modifications, CDK phosphorylation occurs at low levels during the cell cycle (Talasz et al. 1996). H1.1 has an intermediate binding affinity for mononucleosomes and naked DNA (Talasz et al. 1998).

H1.2 (H1c)

This subtype has the highest turnover rate and, unlike other histones, transcription was not restricted to S-phase and maintains a constant presence on chromatin as cells become quiescent, differentiated or both (Pehrson and Cole 1982; Higurashi et al. 1987; Dominguez et al. 1992). It appears to be one of two subtypes critical for the functioning of a mammalian cell, since a survey of human tissue cultures has yet to find a cell line that does not express either H1.2 or H1.4 (Meegans et al. 1997). As the previous subtype, H1.2 is phosphorylated at low levels at every stage of the cell cycle (Talasz et al. 1996). Some studies reported an enrichment of H1.2 in soluble chromatin fractions (Huang and Cole 1984; Gunjan et al. 1999). The implication from these studies is that H1.2 would associate with less condensed regions of chromatin. Overexpression of H1.2 in fibroblast cells did not dramatically increase chromatin compaction, did not interfere with cell cycle progression and did not repressed transcription (Brown et al. 1996; Gunjan et al. 1999). In contrast, there was increased expression of some genes (Gunjan and Brown 1999). Immunolocalization studies in nuclei showed not completely consistent results, a uniformly distribution of H1.2 in human nuclei was described in human fibroblasts by Parseghian (Parseghian et al. 2000), while Hendzel group showed an enrichment of H1.2-GFP fusion in euchromatic regions in neuroblastoma cells (Th'ng et al. 2005). It is difficult to compare studies done in different cell types, which can vary massively in their H1 contents, and also effects of the GFP-tag or of the ectopic expression can not be excluded.

H1.3 (H1d)

This subtype seems to be expressed at very low levels and it belongs to the group of subtypes whose expression decreases in most quiescent and differentiating cells. It has a peak of transcriptional expression at S-phase and is highly phosphorylated at its CDK sites during mitosis (Higurashi et al. 1987; Lenox and Cohen 1983; Talasz et al. 1996). H1.3 appears to be very effective in aggregating nucleosomal arrays (Talasz et al. 1998) and immunoprecipitation experiments showed a selective depletion of the subtype in actively transcribed chromatin

(Parseghian et al. 2000, 2001). On the contrary, nuclear immunostaining studies by Hendzel group have localized H1.3 with euchromatic regions (Th'ng et al. 2005).

H1.4 (H1.4e)

As indicated previously, H1.4 appears to be critical for the functioning of mammalian cells, since a survey of human tissue cultures has not identified a single cell line devoid of H1.4 (Meergans et al. 1997). It remains at an elevated level on chromatin as cell become quiescent, differentiate or both. This subtype is also highly phosphorylated during mitosis (Talasz et al. 1996). It is the only subtype identified to undergo cAMP dependent phosphorylation *in vivo* as well as *in vitro* (Ajiro et al. 1990). The reason for this selective modification, despite the presence of identical sites in H1.2 and H1.3, remains an open question. It is speculated that a rapid reduction in condensation requires weakening of H1.4 binding to bulk chromatin trough phosphorylation mechanism. Some studies suggest that this binding is stronger than that of most other subtypes. H1.4 has a high binding affinity to mononucleosomes and is very effective aggregating nucleosomal arrays (Talasz et al. 1998). Expression of this subtype into cultured cells has led to transcriptional repression of reporter genes. The available data suggest that H1.4 is associated with inactive and condensed chromatin. Inmunoflurescence experiments indicate that H1.4 subtype is preferentially located in heterochromatin (Th'ng et al. 2005).

H1.5 (H1b)

H1.5 belongs to the group of subtypes whose expression declines in differentiated and quiescent cells. This variant has the highest CDK phosphorylation levels at each stage of the cell cycle beginning in G1 (Talasz et al. 1996). It possesses the lowest binding affinity to mononucleosomes and immunoprecipitation studies have determined the presence of H1.5 on actively transcribed chromatin (Parseghian et al. 2000, 2001). However, nuclear distribution data suggests that is located in heterochromatin (Th'ng et al. 2005).

H1.0

This subtype is a differentiation-specific variant and has a very divergent sequence in the globular domain and tails when compared with the other members (Albig et al. 1997).

H1t (testis-specific), H1t2 and HILS1.

They exhibit some variation in the globular domain with a greater variability in the tails when compared to the somatic subtypes (Albig et al. 1997). The testis-specific histone genes are known to be transcribed only in pachytene primary spermatocytes during spermatogenesis (Koppel et al. 1994).

H1oo (oocyte-specific)

Very little is known about this subtype. It seems to be a replacement H1, the expression of which is restricted to the growing/maturing oocyte and to the zygote. It is the only variant whose gene has introns and the transcript gives rise to two distinct, alternatively spliced, mRNA species (H1ooalpha and H1oobeta) (Tanaka et al. 2005).

H1x

H1x was described recently and very little is known. It seems to be the most divergent variant and accumulates in the nucleoli in G1 phase (Happel et al. 2005).

2.3- Linker histone H1 in transcription.

The compaction of chromatin is associated to the inhibition of transcription. As histone H1 contributes to the compaction of chromatin, it was for a long time thought to have a global inhibitory effect in transcription. Although transcriptionally active chromatin is typically depleted in H1 compared with inactive chromatin, the concept of H1 as a general repressor of chromatin activity has been challenged by *in vivo* experiments in which H1 has been overexpressed or depleted. In fact, H1 can also participate to the complex mechanism of gene expression regulation. Several *in vivo* studies have shown that function of linker histone on transcription is gene specific and can have either a positive or negative effect.

In *Xenopus laevis* embryos, Bouvet P. demonstrated that overexpression and incorporation into chromatin of somatic H1 variant repressed oocyte- but not somatic-type 5S rRNA gene or other PolIII transcripts through the modulation of chromatin structure (Bouvet *et al.* 1994; Kandolf 1994; Sera and Wolffe 1998; Vermaak et al. 1998). This is also consistent with what was observed in *Tetrahymena*: the deletion of H1 leads to specific gene activation without a pronounced effect on global transcription by Pol II or Pol III (Shen and Gorovsky 1996).

Several studies conducted on the Mouse Mammary Tumor Virus (MMTV) promoter after hormonal induction have shown a complex role of histone H1 in transactivation of this promoter by steroid hormone (glucocorticoid or progesterone) receptors in human cells. Gunjan *et al.* observed that overexpression of H1.0 and H1.2 mouse isoforms increase both the basal and hormone-induced levels of the MMTV reporter gene transcript (Gunjan et al. 1999). Banks et al. have identified three H1 isoforms that are responsive to hormone exposure in mouse cells. After prolonged dexamethasone exposure, the phosphorylation level of mouse H1.3, H1.4 and H1.5 isoforms decreases, whereas no change is observed in the phosphorylation status of mouse isoforms H1.0, H1.1 and H1.2. Concomitantly, the MMTV promoter is inactivated (Banks et al. 2001).

It is not well known whether the different variants have specific roles or regulate specific promoters. Gene inactivation experiments in mice have shown that elimination of any one of several of these subtypes does not disrupt normal development, although it causes changes in specific gene expression. Deletion of one or two H1 subtypes results in a compensatory upregulation of other subtypes, resulting in a normal level of H1 per nucleosome, and no apparent phenotype (Fan et al. 2001). This suggests that H1 variants have a redundant role in chromatin. Knocking out additional subtypes creates a situation in which the compensation process fails. Triple H1.2-H1.3-H1.5 null embryos die at E11.5 (Fan et al. 2003). Mouse

embryonic stem cells derived from the triple KO showed a 50% of reduction in the normal amount of H1 and only 0.56% of over 4500 genes examined showed significant differences in expression. Among these genes, imprinted genes and sex-chromosomes genes are overrepresented. These genes are normally regulated by DNA methylation in their regulatory regions, and these specific CpG regions are down-methylated in the absence of H1. This suggests that H1 govern an epigenetic regulation of gene expression by contributing to the establishment of specific DNA methylation patterns (Fan et al. 2005). Apart from this, the Nucleosomal Repeat Length (NRL) is strongly reduced. The NRL is one feature of chromatin. There is a wide range of NRL between cell types within an organism (Woodcock et al. 2006). H1 increases the NRL to probably facilitate the more complex organization of chromatin. Fan and collaborators have shown a strong linear relationship between NRL and the ratio H1/nucleosome. Moreover, these triple null ES cells show a decreased in acetylation of K12-H4 and trimethylation of K27-H3. Reductions in both modification levels and NRL may have an additive affect in compensating for H1 loss.

Recently, it has been also reported specific functions for some variants in other processes such as apoptosis or heterochromatinization. The H1.5 variant cooperates with the transcription factor Msx1 for its recruitment to specific target promoters related to myogenesis (Lee et al. 2004). It has been also described an involvement of histone H1.2 in apoptosis induced by DNA double-strand breaks. H1-2 acts as a cytochrome c-releasing factor that appears into the cytoplasm after X-ray irradiation (Konishi et al. 2003). On the other way, SirT1, an NAD+-dependant histone deacetylase involved in establishing repressive chromatin, interacts with and deacetylases K26 of H1.4 (Vaquero et al. 2004). Subsequent methylation of this residue is involved in the recruitment of Polycomb complexes (Kuzmichev et al. 2004), and probably HP1, whereas simultaneous phosphorylation of Ser27 blocks HP1 binding (Hale et al. 2006; Daujat et al. 2005).

Table 4. A compendium of somatic subtype characteristics (reviewed by Parseghian and Hamkalo 2001).

H1.2	H1.3	H1.5	H1.4	H1.1
Distribution in chromatin				
Fractionation of chromatin into soluble and aggregating fragments reveals differential subtype distributions				
Enriched in soluble chromatin				
Fractionation of chromatin after transfection of fibroblasts with H1-Green Fluorescent Protein fusion constructs				
Enriched in soluble chromatin				
Immunolocalization reveals differential subtype distributions within the nucleus				
		Colocalization of $H1^S$-3 PO_4 with RNA processing sites		
Uniform nuclear staining	Speckled nuclear staining		Punctate nuclear staining	
	Colocalization of intense regional staining to the Barr body			
Immunoprecipitation reveals selective depletion of subtypes in chromatin that is actively transcribed or poised for transcription				
	Selective depletion		Selective depletion	
Chromatin condensation and nucleosome-binding studies				
Binding to naked DNA from the MMTV LTR reveals differences in subtype affinities				
High binding affinity	High binding affinity	Lowest binding affinity	High binding affinity	Intermediate binding affinity
Binding to mononucleosomes constructed with DNA from the MMTV LTR also reveal differences in subtype affinities				
High binding affinity	High binding affinity	Lowest binding affinity	High binding affinity	Intermediate binding affinity
There are differences in the capability of subtypes to condense dinucleosomes				
Weakly aggregates dinucleosomes	Strongly aggregates dinucleosomes	Strongly aggregates CTL-3 corresponds to the unresolved mixture of $H1^S$-3 and $H1^S$-4	Strongly aggregates	
There are also differences in the capability of condensing polynucleosomes constructed from the MMTV LTR into chromatin				
Intermediate aggregation into chromatin	Strongly aggregates into chromatin	Weakly aggregates into chromatin	Strongly aggregates into chromatin	Strongly aggregates into chromatin
Overexpression of H1 subtypes in vivo causes...				
No increase in chromatin compaction				
H1 as a transcriptional regulator				
Subtypes reconstituted on H1-depleted chromatin display varying capacities to inhibit RNA polymerase initiation				
33% inhibition of H1-depleted control		66% inhibition of H1-depleted control	75% inhibition of H1-depleted control	
Overexpression of H1 subtypes in vivo leads to diverse transcriptional effects				
Negligible repression of some genes, and increased expression of others				
Increased expression from a transfected MMTV promoter				
				Transcriptional repression of co-transfected promoter sequences
So far, one subtype has demonstrated specific interaction with a promoter element from a core histone gene				
		$H1^S$-3 interacts with the Ω element from histone H3.2		
Subtypes exhibit differential binding to synthetic CpG-rich oligonucleotides and inhibition of DNA methyltransferase activity				
Weak inhibition of Met			Strong inhibition of Met and Preferential binding to CpG	

H1.2	H1.3	H1.5	H1.4	H1.1
Patterns of synthesis				
Synthesis of four subtypes are coupled to S-phase, the fifth expresses both DNA-replication dependent and independent (poly A^+) mRNAs				
Replication independent poly A^+ mRNA	Replication dependent	Replication dependent	Replication dependent	Replication dependent
However, researchers have reported the synthesis of TWO somatic subtypes in proliferating and quiescent cells at the level of proteins and mRNA				
Replication independent			Replication independent	
Differences in the timing of synthesis during S-phase have been reported, while a second group of researchers disagree...				
Starts in mid S-phase Starts in G1-phase	Starts in early S-phase Starts in S-phase	Starts in early S-phase Starts in S-phase	Starts in mid S-phase Starts in G1-phase	Starts in early S-phase Starts in S-phase
One group finds a simultaneous increase of all subtype mRNAs during S-phase, rather, it is mRNA levels that differ. Data from HeLa cells show...				
~10 × H3.3B mRNA levels	~14 × H3.3B	~7 × H3.3B	~11 × H3.3B	
Turnover rates are significantly different for the subtypes in neurons...				
Highest turnover	Second highest turnover	Third highest turnover	Slowest turnover	
...and in lymphomas				
High turnover, half-life = 18 h	Low turnover, half-life = 63 h	Low turnover, half-life = 63 h	High turnover, half-life = 23 h	Low turnover, half-life = 63 h
Subtype quantities change as proliferating cells enter quiescence. Such changes are reflected in developing tissues as an organism matures				
Increase or maintain continued expression	Decrease	Decrease	Increase or maintain continued expression	Decrease
Turnover of subtypes continues in differentiating neurons once cell proliferation has ceased				
			At 70% of total H1, $H1^S$-4 is predominant in mature neurons	
This pattern is also reflected in proliferating vs. quiescent tissue culture cells				
Increase	Decrease	Decrease	Increase	Decrease
Induction of differentiation in murine fibroblasts, myoblasts, and erythroleukaemic cells leads to relative changes in subtype quantity				
Increase or maintain continued expression	Decrease	Decrease	Increase or maintain continued expression	Decrease
However, the pattern seen with most organs does not hold true for lymphoid organs or lymphocytes				
		Still abundant	Remains at low levels	Still abundant
Induction of differentiation in HL60 promyelocytic leukemia cells leads to relative changes in subtype quantity				
Decrease	Slight decrease	Increase	Slight decrease	Not present
Only two subtypes predominate in the chromatin of prepachytene spermatocytes during genetic recombination				
Present in chromatin				Present in chromatin
Synthesis of somatic H1s begins at the late 1-cell stage in mammalian embryos, however, it appears only one subtype exists in 1-cell embryos				
1-cell stage	2-cell stage	2-cell stage	2-cell stage	
Transcriptional analysis of several human cell lines finds two of the subtypes expressed in all of them				
Expressed in all lines			Expressed in all lines	
Structural considerations				
Evolutionary stability of the protein structure differs for each subtype (Lennox studied migration patterns on 2-D gels; Ponte studied sequence data)				
Variable structure Moderately evolving	Conserved structure Moderately evolving	Slightly variable structure Moderately evolving	Conserved structure Slowest evolving	Variable structure Fastest evolving

	H1.2	H1.3	H1.5	H1.4	H1.1
Phosphorylation					
Extent of phosphorylation differs for each subtype during specific stages of the cell cycle					
	Low PO_4 levels at S-phase	High PO_4 levels at S-phase	**Highest PO_4 levels at all stages**	High PO_4 levels at S-phase	Low PO_4 levels at S-phase
Cyclin-Dependent Kinase Phosphorylation sites in mice (rats)					
	4 (4)	5 (5)	5 (5)	4 (5)	4
A hormone-regulated cAMP-Dependent Phosphorylation site at approximately serine 37 does not occur in all variants					
			No serine at residue 37 replaced by threonine		
cAMP-Dependent Phosphorylation in vivo has been observed for only one subtype so far					
	PO_4 occurs in vitro	PO_4 occurs in vitro		PO_4 occurs in vitro and in vivo	
Extent of phosphorylation differs in regenerating rat liver about to undergo DNA replication, with one subtype modified first and foremost					
	Minor levels of PO_4	Not reported	Too small to estimate	PO_4 levels 5× that of normal liver	
Phosphorylation levels may differ for subtypes in transformed fibroblasts					
	Increased PO_4		Increased PO_4		
Apoptotic DNA fragmentation is preceded by varying degrees of dephosphorylation					
	Nearly complete de-PO_4		Mono-PO_4 still present	Mono- and di-PO_4 still present	
Poly(ADP-ribosyl)ation					
There are differences in the extent of modification for subtypes isolated from nuclei that have been incubated with radiolabeled precursors					
	Highest poly(ADPr) content	Not significant	3rd highest Poly(ADPr) content	Not significant	2nd highest Poly(ADPr) content
*There are differences in the extent of modification of subytpes from fibroblast nuclei undergoing poly(ADP-ribosyl)ation-mediated DNA repair**					
	2nd highest poly(ADPr) content	Not reported	Highest poly(ADPr) content	3rd highest poly(ADPr) content	Not present
There are differences in the extent of modification during mitogen activation of lymphoid cells					
	Bulk of poly(ADPr) content				
Gene organization					
The reported number and composition of upstream promoter elements differs among the subtype genes					
	TATA box	TATA box	TATA box	TATA box	TATA box
	CCAATCA box	CCAATCA box	CCAATCA box	CCAATCA box	CCAATCA box
	No Sp1 binding site	Sp1 binding site	Sp1 binding site	Sp1 binding site	Sp1 binding site
	One H1 box	One H1 box	**Two H1 boxes**	One H1 box	**No H1 box**
	Two putative extra H1 boxes				
	CH1UE box	CH1UE box	CH1UE box	CH1UE box	**No CH1UE box**
Upstream of the CH1UE box, GRGTTNGGGGA is a conserved motif and a putative protein-binding site that requires further characterization					
	Sequence is present	Sequence is present	**No sequence**	Sequence is present	**No sequence**

The somatic subtypes are organized into two subclusters at the H1 locus in both mice (chromosome 13 A2-3) and men (6p21.3)

Human 6p21.3 Centromere ⇐ $H1^S$-3 ⇐ ~1.5 Mb ⇒ $H1^S$-2 — $H1^S$-4 — H1t — $H1^S$-1 — H1a ⇒ Telomere

Mouse 13A2-3 Centromere ⇐ $H1^S$-3 ⇐ >500 kb ⇒ $H1^S$-2 — $H1^S$-4 — H1t — $H1^S$-1 — H1a ⇒ Telomere

2.4- Post-translational modifications of linker histone H1.

The tails of H1 variants are subject to multiple posttranslational modifications such as phosphorylation, acetylation and methylation. It remains to be seen how these modifications play a role regulating the transitions between the open and compacted chromatin states and if they are specific depending on the variant involved. Maps of histone H1 variants modifications have been recently established (Garcia et al. 2004; Wisniewski et al. 2007).

H1 phosphorylation

Histone H1 can undergo phosphorylation on specific Ser and Thr residues on N- and C-terminal domains of the molecule. These residues belong to the (S/T)-P-X-(K/R) motif, which are widely spread in H1 terminal tails, and correspond to the cyclin dependent kinase phosphorylation sequence. A multiplicity of cyclin-dependent kinases have been found to phosphorylate mammalian H1 histones at all their growth-related sites but analysis *in vivo* has been complicated by the diversity of the heteromorphous H1 histone variants.

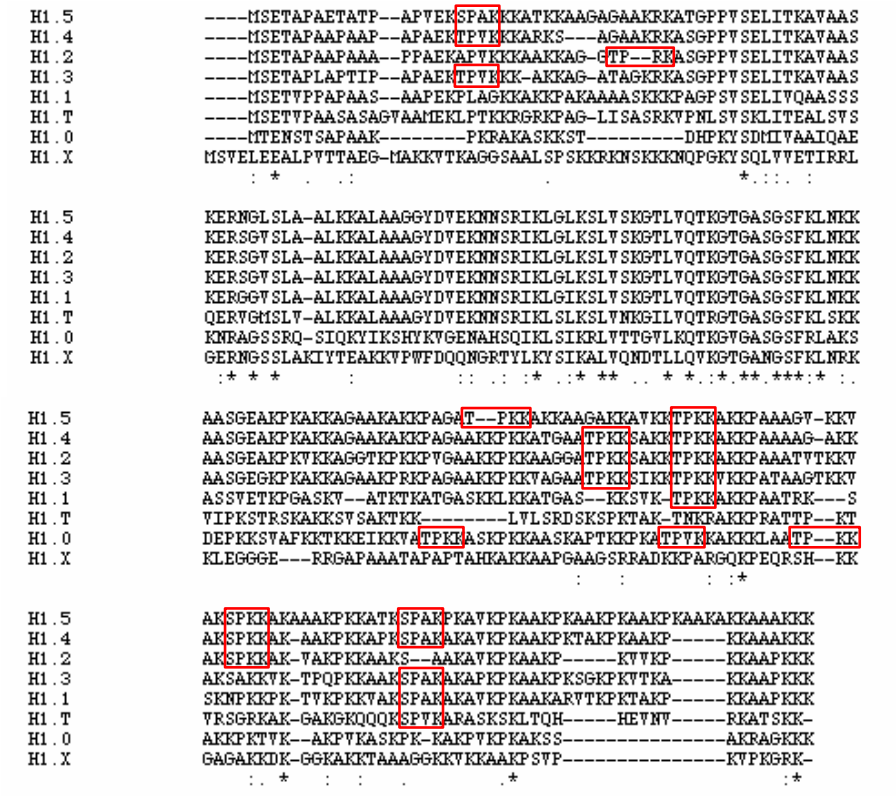


Figure 6. CDK phosphorylation motifs (S/T)-P-X-(K/R) in human H1variants.

The phosphorylation is cell cycle dependent with the highest level in M-phase (Sarg et al. 2006; Talasz et al. 1996; Davie et al. 2004). Paradoxically, in M phase chromatin is highly condensed and phosphorylation of H1 has been also associated with active interphase

chromatin. The consequence of increased H1 phosphorylation appears to be the relaxation of chromatin structure (Chadee et al. 1995; Herrera et al. 1996; Taylor et al. 1995). However, the observation that the highest levels of H1 phosphorylation occurs during mitosis can be explained by the model proposed by Bradbury et al., in which it is suggested that phosphorylation drives chromosome condensation by promoting H1-H1 protein interactions via the globular domains (Bradbury 1992). An alternative model postulates that phosphorylation of H1 weakens tail-DNA interactions and decreases H1-H1 globular domain binding, resulting in a decondensed chromatin state (Roth et al. 1992) necessary for structural reorganization as chromatin condenses into chromosomes during mitosis. Support for the latter model comes from experiments which have demonstrated that while unphosphorylated linker histone inhibits the activity of ATP-dependent chromatin remodelling enzymes on nucleosomal arrays, *in vitro* phosphorylation of the histone before incorporation into the arrays can restore enzyme activity by relaxing the topological constrains induced by unphosphorylated histone H1 (Horn et al. 2002).

H1 acetylation

According to Garcia et al., acetylation of Lys26 of H1.4, Ser2 of most of the somatic variants and Lys62 in H1.4 and its equivalent in H1.2 and H1.3 have been identified by mass spectrometry techniques, although very little is known about the validation *in vivo* (Garcia et al. 2004).

It has been speculated that acetylation of Lys 26 would contribute to the neutralization of H1 highly charged tails, and in this way facilitates chromatin decondensation. SirT1 has been identified as the deacetylase for this residue, as well as, for Lys9 of H3 or Lys16 of H4 (Vaquero et al. 2004). Despite of this, these modifications need further studies.

H1 methylation

Trimethylation of Lys26 of H1.4 by Polycomb Repressive Complex 3 has been identified recently by Dr. Reinberg's laboratory. Human Enhancer of Zeste homolog (Ezh2) is a histone methyltransferase present in PRC2 and PRC3. While studying H3 methylation, this group realized that depending on the isoform of Eed protein present in the complex, it can target its methylase activity towards histone Lys27 of H3 or Lys26 of H1 (Kuzmichev et a 2004). It is still unknown whether other H1 variants can be also methylated, but the region surrounding the methylation site in H1.4 is not conserved among them.

Methylated H1.4 has been shown to interact with all HP1 isoforms through the chromodomain (Daujat et al. 2005). Moreover, phosphorylation of neighbouring Ser27 disrupts

this interaction, as also happens for methyl Lys9 of H3 when Ser 10 is phosphorylated (Fischle et al. 2005), mimetizing the "phospho switch" model proposed by Allis.

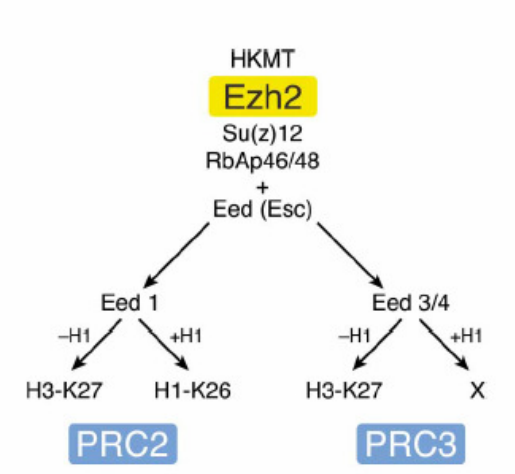


Figura 7. Polycomb repressive complexes described and their substrate specificity (Vaquero et al. 2004).

H1 ADP-ribosylation

Poly(ADP-ribose) is a homopolymer of ADP-ribose units that is synthesized by poly(ADP-ribose) polymerase (PARP) upon activation by DNA strand breaks (Ikejima et al. 1990; D'Amours et al. 1999). The ADP-ribose moieties are transferred from NAD to nuclear proteins that include histone H1, and visualization of isolated chromatin by electron microscope showed that poly(ADP-ribosyl)ation prevented the condensation of polynucleosomes and maintained it in a relaxed conformation under conditions of high ionic strength (Poirier et al. 1982). Early studies showed that this modification may play a regulatory role during apoptosis. Inhibition of PARP activity also inhibited DNA fragmentation and cell death, suggesting that modification of nuclear proteins was required for apoptosis. Yoon et al. (1996) showed that poly(ADP-ribosyl)ation occurred on histone H1 to increase the accessibility of chromatin to nucleases and proposed that this could promote DNA fragmentation during apoptosis.

Table 5. Summary of the identified peptides with posttranscriptional modifications from HeLa and MCF-7 cells (Wisniewski et al. 2007).

Cell line	Variant	Modification[a]	Peptide	Sequence with modificati
Hela	H1.2	pS-2	2-17	a-**pS**ETAPAAPAAAPPAEK
Hela	H1.5	pS-2(pT-4)	2-17	a-(**pS**)E(**pT**)APAETATPAPVEK
MCF7	H1.5	pS-2	2-17	a-**pS**ETAPAETATPAPVEK
MCF7	H1.4	pT-18	2-20	a-SETAPAAPAAPAPAEK**pT**PVK
Hela	H1.2	aK-17	2-21	a-SETAPAAPAAAPPAE**aK**APVK
MCF7	H1.2	aK-17	2-21	a-SETAPAAPAAAPPAE**aK**APVK
Hela	H1.3	pT-18	2-21	a-SETAPLAPTIPAPAEK**pT**PVK
MCF7	H1.3	pT-18	2-21	a-SETAPLAPTIPAPAEK**pT**PVK
MCF7	H1.3	aK-17	2-21	a-SETAPLAPTIPAPAE**aK**TPVK
MCF7	H1.3	pT-18	2-21	SETAPLAPTIPAPAEK**pT**PVK
Hela	H1.4	aK-17	2-21	a-SETAPAAPAAPAPAE**aK**TPVK
Hela	H1.4	pT-18	2-21	a-SETAPAAPAAPAPAEK**pT**PVK
Hela	H1.4	pT-18	2-21	a-SETAPAAPAAPAPAEK**pT**PVK
Hela	H1.4	pS-18	2-21	SETAPAAPAAPAPAEK**pT**PVK
MCF7	H1.4	aK-17	2-21	a-SETAPAAPAAPAPAE**aK**TPVK
MCF7	H1.4	pT-18	2-21	a-SETAPAAPAAPAPAEK**pT**PVK
MCF7	H1.4	pT-18	2-21	SETAPAAPAAPAPAEK**pT**PVK
Hela	H1.5	pS-18	2-21	a-SETAPAETATPAPVEK**pS**PAK
Hela	H1.5	pS-18	2-21	SETAPAETATPAPVEK**pS**PAK
MCF7	H1.5	aK-17	2-21	a-SETAPAETATPAPVE**aK**SPAK
MCF7	H1.5	pS-18	2-21	a-SETAPAETATPAPVEK**pS**PAK
MCF7	H1.5	pS-18	2-21	SETAPAETATPAPVEK**pS**PAK
Hela	H1.1	pS-2	2-22	a-**pS**ETVPPAPAASAAPEKPLAG
Hela	H1.1	aK-22	2-23	a-SETVPPAPAASAAPEKPLAGa
Hela	H1.X	pS-31	24-34	AGGSAAL**pS**PSK
Hela	H1.2; H1.3; H1.4	pS-36	34-46	KA**pS**GPPVSELITK
MCF7	H1.2; H1.3; H1.4	aK-34	34-46	**aK**ASGPPVSELITK
MCF7	H1.2; H1.3; H1.4	pS-36	34-46	KA**pS**GPPVSELITK
Hela	H1.2; H1.3; H1.4	ubK-46	34-52	KASGPPVSELIT**ubK**AVAASK
Hela	H1.2; H1.3; H1.4	aK-46	35-52	ASGPPVSELI**TaK**AVAASK
MCF7	H1.2; H1.3; H1.4	aK-46	35-52	ASGPPVSELI**TaK**AVAASK
MCF7	H1.5	aK-49	37-55	KATGPPVSELI**TaK**AVAASK
MCF7	H1.5	aK-49	38-55	ATGPPVSELI**TaK**AVAASK
Hela	H1.2; H1.3; H1.4	aK-52	47-54	AVAAS**aK**ER
Hela	H1.2; H1.1; H1.3; H1.4	aK-64	64-75	**aK**ALAAAGYDVEK
Hela	H1.2; H1.1; H1.3; H1.4	aK-85	82-90	LGL**aK**SLVSK
MCF7	H1.5	fK-88	85-93	LGL**fK**SLVSK
MCF7	H1.5	aK-88	85-93	LGL**aK**SLVSK
Hela	H1.2; H1.1; H1.3; H1.4	aK-90	86-97	SLVS**aK**GTLVQTK
Hela	H1.2; H1.1; H1.3; H1.4	fK-90	86-97	SLVS**fK**GTLVQTK
MCF7	H1.2; H1.1; H1.3; H1.4	aK-90	86-97	SLVS**aK**GTLVQTK
MCF7	H1.2; H1.1; H1.3; H1.4	aK-97	91-106	GTLVQT**aK**GTGASGSFK
Hela	H1.5	pT-138	133-141	KPAGA**pT**PKK
MCF7	H1.5	pT-138	133-141	KPAGA**pT**PKK
Hela	H1.2	pT-146	140-149	KAAGGA**pT**PKK
Hela	H1.3	pT-147	141-149	KVAGAA**pT**PK
MCF7	H1.3	pT-147	141-149	KVAGAA**pT**PK
MCF7	H1.2	pT165	160-169	KPAAA**pT**VTKK

3. Steroid hormone receptors.

3.1- Common features for steroid hormone receptors (SHRs).

The nuclear receptors are ligand activated transcription factors that regulate the expression of target genes. The nuclear receptor superfamily is subdivided into three families (Chawla et al. 2001):

a) the steroid receptor family;

b) the thyroid/retinoid family, that includes thyroid receptor (TR), vitamin D receptor (VDR), retinoic acid receptor (RAR), and peroxisome proliferator-activated receptor (PPAR);

c) the orphan receptor family, which ligands are unknown.

The gonads and adrenal gland produce five major groups of steroid hormones (SHs): estrogens, progestins, androgens, glucocorticoids and mineralocorticoids. SHs have importantly regulatory roles in a wide variety of biological processes including reproduction, differentiation, development, cell proliferation, apoptosis, inflammation, metabolism, homeostasis and brain function (Mangelsdorf et al. 1995, Tsai and O'Malley 1994).

SHs are small lipophilic molecules that go through the cell membrane by simple diffusion and bind to steroid hormone receptors (SHRs). Unliganded SHRs are associated with a large multiprotein complex of chaperones that keep indefinitely the SHRs folding process, in order to repress SHRs transcriptional activity until the receptor is activated by their corresponding SHs (Pratt 1993, Smith 2000). Upon SHs activation, SHRs act as transcription factors and modulate gene expression.

There have been described and characterized at least one receptor per each steroid hormone known and in some cases several isoforms: GR (glucocorticoid receptor), ERalpha and ERbeta (estrogen receptor), AR (androgen receptor), PRA and PRB (progestin receptor) and MR (mineralocorticoid receptor). All of them contain two structural subunits (Figure 8):

a) a C-terminal ligand binding domain (LBD)

b) a centrally located DNA-binding domain (DBD)

Figure 8. Steroid hormone receptor (SHR) structure.

The LBD contains a specific pocket for its corresponding hormone and a ligand-regulated transcriptional activation function (AF-2) necessary for recruiting co-activating proteins. This region is also the primary mediator of dimerization, necessary for DNA response element binding (Kumar and Chambon 1988).

The DBD is the responsible of the binding to the DNA, in particular to hormone responsive elements (HREs) located in those genes responding to hormone. DBD is also an allosteric transmitter of information to other regions of the receptor molecule (Beato and Klug 2000).

Both domains are connected by a short amino acid sequence called the hinge. The functional properties of the hinge region are still unclear, although it can be phosphorylated and this phosphorylation is coupled to increased transcription activation (Knotts et al. 2001, Vicent el al. 2006).

The N-terminal part of the receptors contains a transcriptional activation function called AF-1. AF-1 sequence shows weak conservation between the SHRs family members. This could explain how closely related SHRs can bind to similar response elements *in vitro*, but differentially regulate promoter containing those sequences *in vivo* (Takimoto et al. 2003).

3.2-Progesterone receptor (PR).

PR belongs to the steroid receptor family of nuclear receptors and mediates the action of the progesterone hormone, a key ligand in reproduction and pregnancy. As it has been described previously, progesterone binds to a pocket in the LBD triggering the ligand-dependent activation function (AF-2) of the receptor (Figure 9) and recruiting coactivators such as the steroid receptor coactivator (SRC) family (Xu and Li 2003). PR binds to the DNA as a dimer through its DBD composed of two different zinc-finger structures. Upon binding to a palindromic response sequence, PR domains suffer conformational changes localized into the AF-1 and the hinge region. These changes seem to be also necessary for recruitment of coactivators to the target promoter (Kumar and Thompson 2005).

The progesterone receptor (PR) was the first SHR shown to exist in two isoforms generated by differential promoter usage (Kastner et al. 1990). The longer form is called PRB while the shorter form, PRA, lacks 164 aa in its N-terminal domain (Beato, Klug 2000). Although both isoforms show a high degree of sequence identity and structure, they display significant functional properties on the regulation of target promoters. Microarray studies showed that the two isoforms regulate different subset of genes (Richer et al. 2002), being PRB a much stronger transcriptional activator than PRA (Sartorius et al. 1994).

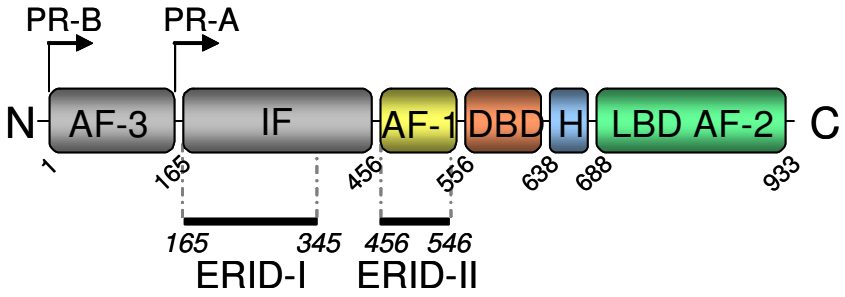


Figure 9. Progesterone receptor (PR) structure.

3.3.- SHRs direct effects.

Nuclear translocation of the SHRs is a SHs dependent process mediated by several nuclear localization signals (NLS) located in the C-terminus of the second zinc finger, in the hinge region and in the LBD. At equilibrium, the majority of SHRs are in the nucleus due to the presence of these NLSs required for nuclear pore recognition (Guiochon-Mantel et al.1991).

In the nucleus, PR, GR, MR and AR bind to the same HREs (Figure 10). HREs are composed of hexanucleotides (TGTTCT) arranged as inverted repeats (palindromes) and separated by three non-conserved base-pairs (Beato 1989; Truss et al.1991). Each half-site is recognized by one receptor monomer (Luisi et al. 1991). DNA nucleotide sequence and its specific packaging in chromatin determine the interaction of hormone regulatory sites with their correspondent SHR (Beato, Klug 2000).

In order to regulate transcription, agonist-liganded SHRs have to interact with the members of the general transcription machinery in order to be recruited to the chromatin-organized promoters. SHRs can interact directly with general transcription factors or with coactivators or mediators (Beato, Klug 2000). In this way, SHRs can also activate genes lacking HREs by interaction with other sequence-specific transcription factors bound to their target sequences (Beato, Herrlich and Schutz 1995).

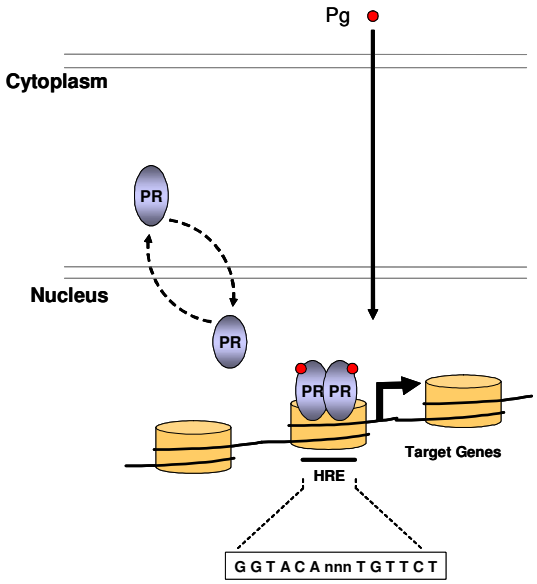


Figure 10. Direct-mediated effects of PR.

3.4.- Signalling mediated effects.

In addition to the genomic effects, SHs induce rapid responses of kinase cascades activated by cytoplasmic events. These rapid, steroid "non-genomic effects", often occur through signalling complexes located at membranes. Consequently, receptors for signalling-mediated actions are widely believed to be membrane-associated or integrated into the membrane (Wehling, Losel 2006).

Estrogens activate the Src/Ras/Erk and the PI3K/Akt pathways by direct interaction of ER with SH2 domain of c-Src and the regulatory subunit of PI3K, respectively (Castoria et al. 2001, Migliaccio et al. 1996). These pathways are determinant for estrogen induction of cell proliferation in breast cancer cells.

Progesterone also crosstalks to kinase cascades trough an interaction of PR with SH3 domain of c-Src (Ballare et al. 2003, Boonyaratanakornkit et al. 2001). In breast cancer cells, containing ER, the progesterone effect on the Src/Ras/Erk pathway is mediated by the interaction of two N-terminal PR domains (ERID I and II) with LBD of ERalpha, which itself triggers the activation of the cascades (Ballare et al. 2003, Migliaccio et al. 1998). The targets of the activated kinase cascade might be transcription factors, other kinases, histones and co-regulators involved in gene expression, and ultimately responsible of some hormone effects such as DNA synthesis and cell proliferation (Figure 11).

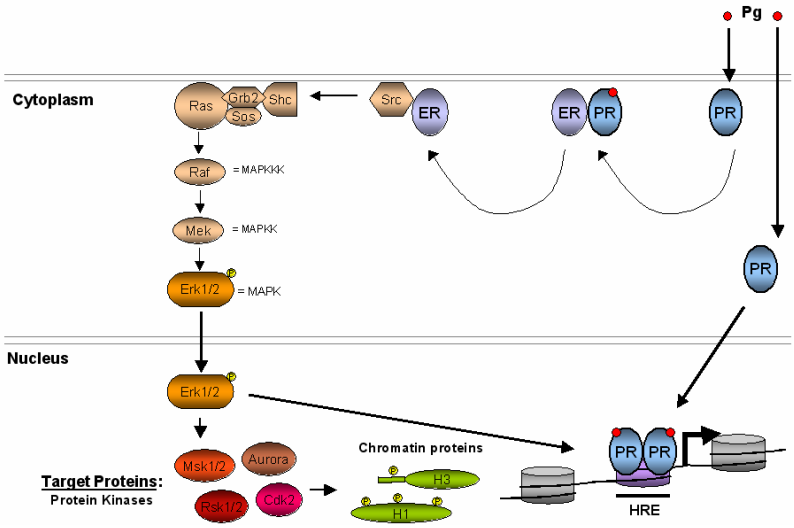


Figure 11. Signalling mediated effects of PR.

3.5.- SHRs and transcription: PR regulation of the MMTV promoter and the 11β-HSD2 promoter.

The mechanisms involved in activation of gene transcription by steroid hormone receptors are still not clear. *In vitro* transcription assays showed that ligand-activated receptor increase the rate of transcription by recruiting and stabilizing the pre-initiation complex at the promoter of hormone responsive target genes (Beato, Sanchez-Pacheco 1996). Thus, *in vitro* binding of SHRs to general transcription factors that associate with RNA polymerase II has been described as a possible mechanism for recruitment of the pre-initiation complex. *In vivo*, the fact that SHRs regulate transcription through interaction with HREs that function as enhancers, suggests the existence of other mechanisms for receptor communication with basal transcriptional machinery, for instance the recruitment of coactivators, corepressors, general transcription factors, chromatin remodelling factors and post-translational modifications of histone-tails (Figure 12).

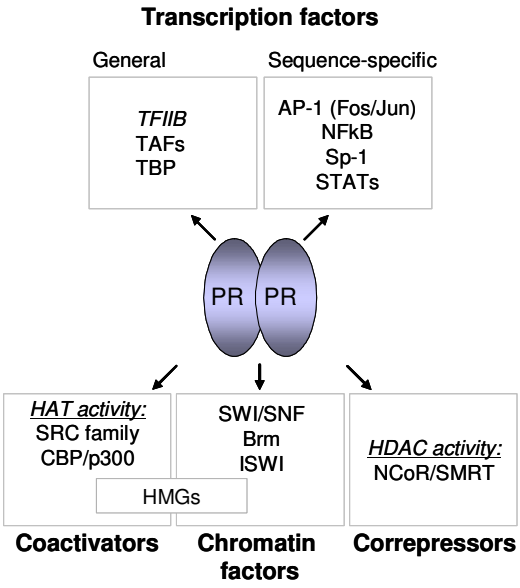


Figure 12. Overview of nuclear partners of PR.

Many regulatory regions of hormone-responsive genes are organized in positioned nucleosomes, which are remodelled in the context of hormone induction. One of the most extensively studied model systems is the hormone regulation of the Mouse Mammary Tumour Virus (MMTV) promoter.

The MMTV promoter is organized in positioned nucleosomes (Figure 13), with the nucleosome B covering the HREs region and a binding site for NF1 (Richard-Foy, Hager 1987). For hormone activation of the promoter is required not only the HREs but also the NF1 binding site, indicating that both factors, PR and NF1, synergize *in vivo*.

The model proposed for the hormone activation of the MMTV includes the collaboration of both genomic and non-genomic effects. Thus, progestins stimulate proliferation of human breast cancer cell lines and a rapid activation of the Src/Ras/Erk pathway that requires PR and ER (Ballare et al. 2003). Progestin activation of the PR/ER complex triggers ER-mediated activation of the Src/Ras/Erk cascade and accumulation of active Erk1/2 in the nucleus (Vicent et al. 2006). Ligand binding to the complex of PR in the nucleus leads to the formation of PR homodimers that are phosphorylated by activated Erk1/2, which also phosphorylates Msk1. The three activated proteins form a terniary complex that is recruited to the nucleosome B, due to the affinity of PR for the exposed HREs. Once bound to the promoter, Msk1 phosphorylates histone H3S10 generating a signal that leads to displacement of a repressive complex containing HP1gamma. Thus, ATP-dependent chromatin remodelling complexes are

recruited by PR and removal of H2A/H2B dimmers from nucleosome B allows binding of NF1, coactivators, displacement of H1 and recruitment of the basal transcriptional machinery including RNA polymerase II (Vicent et al. 2006).

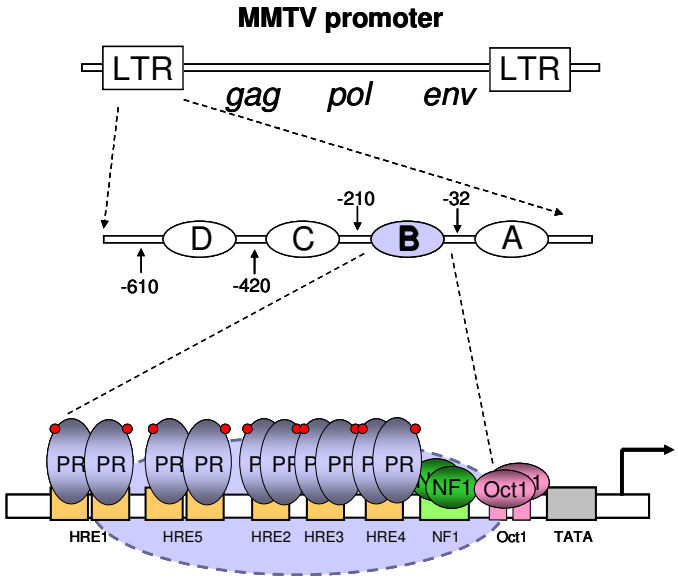


Figure 13. Schematic representation of the nucleosome structure of MMTV promoter and the main binding sites in nucleosome B.

In vitro nucleosome assembly with MMTV promoter DNA, showed that the promoter adopts a precise rotational orientation on the surface that exposes HRE-1 and HRE-4, but leaves inaccessible the central HRE-2, -3 and –5 and the NF1 binding site, essential for hormone induction (Eisfeld et al. 1997, Piña et al., 1990). These evidences showed that the nucleosome may undergo changes during hormone induction to enable the binding of PRs and NF1 and their synergism. NF1 seems to be necessary to stabilize the "open" conformation of the nucleosome and facilitate the access of PR (Di Croce et al. 1999).

It has been also described that dynamics of the nucleosome could be also regulated by linker histone H1. In the presence of H1, SWI/SNF complex cannot remodel nucleosomes *in vitro* (Horn et al. 2002). H1 binds asymmetrically to MMTV nucleosomes, with preference for the distal 5' end (Vicent, Melia et al. 2002). MMTV promoter transcription and induction by PR and NF1 were enhanced in H1 containing minichromosomes, due to the better positioning of nucleosomes in the presence of H1 and a better binding of PR (Koop et al. 2003). In the presence of bound PR, H1 is phosphorylated and removed from the promoter on transcription initiation (Figure 14).

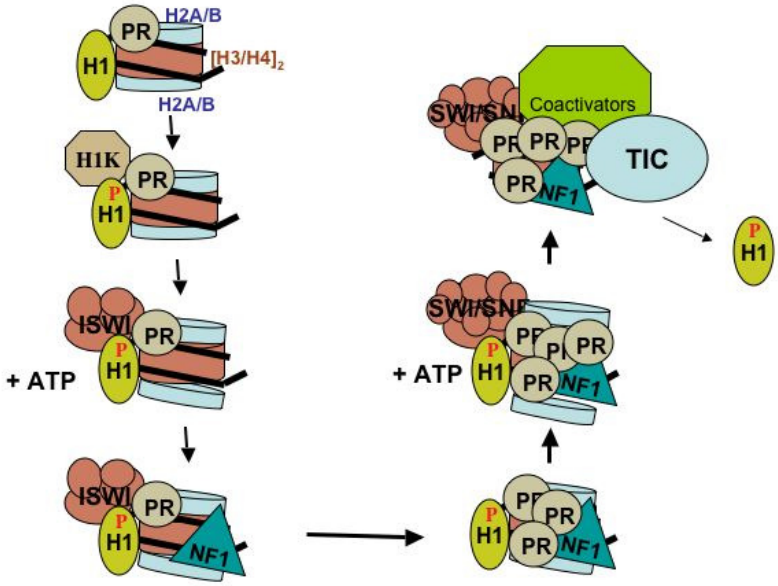


Figure 14. Model proposed for PR-mediated activation in the MMTV.

The 11b-HSD2 is the endogenous model in the laboratory for studying progesterone gene regulation in breast cancer cells. The distal region of the promoter, between -1178 and -1345, has been defined as the minimal part of the promoter regulated by hormone. The rapid mechanism of activation in this case is mediated by another kinase pathway: JAK/STAT signalling. Chromatin immunoprecipitation experiments showed a STAT5A-mediated recruitment of PR to the distal promoter upon hormone treatment, impaired by JAK/STAT pathway inhibitors (Subtil-Rodríguez et al 2008). This promoter together with the MMTV will be use for studying specific participation of H1 subtypes upon hormone induction.

Forty years ago, Kinkade and Cole described the first biochemical fractionation of histone H1 variants (Kinkade et al. 1966), yet the specific functional role of individual H1 is still not fully understood.

The presence of different H1 variants could simply be due to polymorphism, that arose after gene duplications during evolution. According to this hypothesis the timing of expression of the variants would have more functional relevance than amino acid differences in the proteins themselves. The viability of many H1 knockouts in mice and the compensation by other H1 variants seems to support this hypothesis. However, there are now many evidences favouring a specific function for H1 variants. First, the primary sequence of the H1 variants is moderately conserved between animal species. Estimation of the rates of nucleotide substitutions during evolution points towards a functional differentiation, since the rate of nucleotide substitutions differed among them (Ponte et al. 1998). Second, careful analysis of individual H1 variant knockout mice revealed specific phenotypes (Gabrilovich et al. 2002) and distinct effects on gene expression and chromatin structure (Alami et al. 2003). Third, in mice with single H1 knockout not all of the remaining H1 variants are up-regulated, but only specific subtypes are able to compensate. Fourth, differences in the intracellular localization of H1 variants has been found (Parseghian et al. 2000; Th'ng et al. 2005) and their relative composition varies between cell types (Meergans et al. 1997). Fifth, H1 variants bind to chromatin with different affinities (Th'ng et al. 2005; Orrego et al. 2007). Sixth, specific H1 variants can be recruited by transcription factors to unique binding sites (Lee et al. 2004). Seventh, the H1 variants differ in their post-translational modification pattern (Sarg et al. 2006).

The functional heterogeneity of linker histone H1 is an interesting field of research. *In vivo* studies with particular focus on individual H1 subtypes as the ones that we will present in this thesis will be essential to decipher the functionality of this conserved class of highly abundant nuclear proteins.

OBJECTIVES

The linker histone in mammals is a family of different histone H1 variants, including five somatic subtypes (H1.1 to H1.5) and the maintenance variant H1.0, in addition to some tissue-restricted subtypes. It is poorly understood why so many H1 variants exist and whether they have particular functions.

The general objective of the current thesis was to further characterize *in vivo* the specificity of human histone H1 variants in chromatin structure, gene expression and cell proliferation. Particular objectives developed along the thesis are:

1. generation of tools to study the role of the different somatic variants (H1.2 to H1.5) and the replacement H1.0 subtype:
    - production of specific antibodies for each variant.
    - generation of constitutive and inducible H1 variant knock-down cell lines using RNA interference.
    - generation of cell lines expressing HA-tagged H1 subtypes.
2. study of the properties of the different H1 variant knock-down cell lines. Characterization of the depleted cell lines in terms of level of inhibition, compensatory effects, cell proliferation rates and morphology alterations.
3. analysis of the consequences of H1 variant knock-down on gene expression. Participation of H1 variants on basal gene expression and also in response to an external stimulus such as steroid hormones.
4. examination of changes on chromatin structure and epigenetic marks. For instance, alterations in nucleosome spacing or variations in core histone modifications.
5. study whether it exists a specific participation of any particular H1 variant(s) in the regulation of gene promoters, in particular hormone responsive promoters studied in the group.
6. investigation of the specificity of H1 variants distribution in the nuclei.

MATERIAL & METHODS

1. Plasmids.

Plasmid pSuperRetro was a gift from Dr. Mike Edel. It is a MoMuLV-derived retroviral vector that includes a puromycine selection marker. The oligonucleotides for shRNAs were cloned into the vector through BglII and HindIII restriction sites.

pLVTHM and ptTR-KRAB-Red were a gift from Dr. Didier Trono, all of them lentiviral HIV-derived vectors containing GFP and dsRed fluorescence markers, respectively. ShRNAs oligonucleotides were introduced in the pLVTHM plasmid through ClaI and MluI restriction sites.

pVsVg from Clontech, is a plasmid codifying for viral envelope proteins, and pCMVΔR8.91 from Dr. Didier Trono codifies for viral *gag* and *pol* genes, both necessary for the production of viral particles in the case of lentiviral vectors.

$pCDNA_4$ vector from Clontech, is an extensively used vector for expression of exogenous proteins. Somatic H1 variants were cloned by PCR amplification from genomic DNA with specific primers containing EcoRI restriction site. PCR products were cloned in pGEMT vector according to manufacture's instructions (Invitrogen). This system is used as intermediate vector for digesting with restriction sites of interest. Digestion product was cloned in $pCDNA_4$ – HA plasmid (already containing the HA tag) through EcoRI restriction site upstream the tag.

pEV833 is a lentiviral HIV-derived vector from Dr. Eric Verdin. It has been used to generate stably HA-tagged H1 variant expressing cell lines. The HA-tagged H1 variants were cloned from the previous vector $pCDNA_4$ through SmaI restriction site.

All the viral resulting plasmids were transformed into electrocompetent *E.coli* STBL2 bacteria. Positive clones were selected by restriction analysis and sequencing. STBL2 Competent cells (Gibco) are suitable for the cloning and maintenance of unstable vectors as retroviral sequences. Bacteria growth should be done at 30ºC.

Non viral plasmids are always transformed in competent *E.coli* DH5α bacteria and cultured at 37ºC.

2. Oligonucleotides.

shRNAs oligonucleoides

The 64 mer oligonucleotides for histone H1 shRNA were designed with the OligoEngine Workstation2 program and obtained from Sigma at 0.05µM scale. Annealing and phosphorylation protocols of the oligonucleotides were performed as suggested by Oligoengine protocol. The target sequences tested for each H1 subtype are in the following table. The working shRNAs are indicated in red.

	Sequence position in mRNA	Target Sequence
H1.0	54	GGCCTCCAAGAAGTCCACA
	104	CCATCCAGGCCGAGAAGAA
	190	**CGCTGACTCGCAGATCAAG**
	290	GAGCGACGAACCCAAGAAG
H1.2	51	GGCCCCTGTAAAGAAGAAG
	156	**AGAGCGTAGCGGAGTTTCT**
	379	ACCTAAGAAGCCAGTTGGG
	436	CTCCGAAGAAGAGCGCTAA
H1.3	73	GAAGGCAGGCGCAACTGCT
	88	CTGCTGGGAAACGCAAAGC
	140	GGCAGTGGCAGCTTCTAAG
	473	GGTAAAGAAGCCAGCAACC
	491	CCGCTGCTGGGACCAAGAA
	506	AAAGTGGCCAAGAGTGCGT
	555	**CTGCCAAGAGTCCAGCTAA**
H1.4	120	GTCCGAGCTCATTACTAAA
	225	GAACAACAGCCGCATCAAG
	444	**GAAGAGCGCCAAGAAGACC**
	458	GACCCCAAAGAAGGCGAAG
H1.5	75	**GGCAACTAAGAAGGCTGCC**
	165	GGAGCGCAATGGCCTTTCT
	371	GAAGGCAGGCGCCGCTAAA
	419	GAAGGCCAAGAAGGCTGCA
	562	AAGAGTCCTGCCAAGCCCA

Oligonucleotides for ChIP

	Name	Sequence from 5' to 3'
MMTV	Nuc B up	GGGCTTAAGTAAGTTTTTGGTTACA
	Nuc B low	TTTACATAAGATTTGGATAAATTCC
11β-HSD2	11β-up (-1778)	GGGGTGCTGTGTCTGCCTCCAAG
	11β-low (-1596)	GCCATGACCCTGTGTGTGCAAGT
β-globin	up	ACACAACTGTGTTCACTAGC
	low	CAACTTCATCCACGTTCACC

Oligonucleotides for RT-q-PCR

		Sequence from 5' to 3'
GAPDH	up	TTGGTCGTATTGGGCGCCTGG
	low	CAAAGTTGTCATGGAT
CDKN1A	up	CAGGGGACAGCAGAGGAAGA
	low	GGGCGGCCAGGGTATGTA
CDC2	up	CTGGGGTCAGCTCGTTACTC
	low	AGGCTTCCTGGTTTCCATTT
CDC20	up	CTACAGCCAAAAGGCCACTC
	low	AGAGCTTGCACTCCACAGGT
CDKN3	up	CATAGCCAGCTGCTGTGAAA
	low	CCCGGATCCTCTTAGGTCTC
RFC3	up	CAGTGGCACAATCACAACAA
	low	GAACCGCCAAGCACCTACTA
CCND1	up	CCCTCGGTGTCCTACTTCAA
	low	AGGAAGCGGTCCAGGTAGTT
CCNB2	up	TTGCAGTCCATAAACCCACA
	low	GAAGCCAAGAGCAGAGCAGT
MAD2L1	up	CTTCTCATTCGGCATCAACA
	low	TCCAGGACCTCACCACTTTC
H1.0	up	CCTGCGGCCAAGCCCAAGCG
	low	GCTGACTCGCAGATCAAGTT
H1.2	up	GGCTGGGGGTACGCCT
	low	GGGCGGAACCAAACCTAA
H1.3	up	CTGCTCCACTTGCTCCTACC
	low	GCAAGCGCTTTCTTAAGC
H1.4	up	GTCGGGTTCCTTCAAACTCA
	low	CTTCTTCGCCTTCTTTGGG
H1.5	up	CATTAAGCTGGGCCTCAAGA
	low	TCACTGCCTTTTTCGCCCC
LUCIFERASE	up	CTAGAGGATGGAACCGCTGG
	low	ACACCGGCATAAAGAATTGAAGA

3. Reagents.

Doxyciline (Sigma) was dissolved in water and used at 2.5 µg/µl; Propidium Iodide (Sigma) was use at 10mg/ml; Methyl methane- sulfonate (MMS) and hydroxyurea (HU) were purchased from Sigma and used both at 200 µM. Puromycine (Sigma) was used at a final concentration of 2ug/ul to select clones infected with pSuper.Retro vector.

R5020 was purchased from PerkinElmer Life Sciences, dissolved in ethanol 100% and used at physiological concentration (10^{-8}M).

4. Antibodies.

ANTIBODIES	COMPANY	
Total H1 AE-4	Upstate	05-457
Phospho-H1	Upstate	06-597
H1.0	Abcam	ab11079
H1.2	Abcam	ab4086
H1.2	Abcam	ab17677
H1.3	Abcam	ab17679
H1.5	Abcam	ab18208
pT146	Abcam	ab3596
pT18	Abcam	ab3595
pT60	Abcam	ab4269
Phosho-H1	Abcam	ab4270
HA	Abcam	ab9110
3meK9-H3	Abcam	ab8898
3meK27-H3	Reinberg's lab	-
acK12-H4	Abcam	ab1761
p27 (C-19)	Santa Cruz	sc-528
p21 (C-19)	Santa Cruz	sc-397
pRb (Ser 608)	Cell Signalling	2181
Cdc2	Abcam	ab18
Tubulin	Sigma	-
SirT1	Reinberg's lab	-
Ezh2	Reinberg's lab	-
H3	Abcam	ab1791
H4	Abcam	ab10156
1meK27-H3	Reinberg's lab	-

Home-made H1 antibodies were generated in the laboratory as described below.

Production of H1 variant specific antibodies.

Peptides were synthesized and conjugated to a carrier protein, Keyhole Limpet Hemocyanin (KLH), in the Proteomic Facility of the Pompeu Fabra University. Each of two rabbits were injected with 200μg of peptide for each H1 variant, at two-weeks intervals, for a total of 4 immunizations. Sera were collected and analyzed a week after last immunization.

5. Cell culture.

- Cell line HEK 293T, derived from human embryonic kidney, was grown at 37 ºC with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% glutamine (200 mM) and 1% penicillin/streptomycin (10000 unit/ml / 10000μg/ml).
- Cell line GP2.293 was grown at 37 ºC with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS, 2% glutamine and 1% penicillin/streptomycin.
- Cell line T47D-MTVL (8), derived from the human mammary carcinoma cell line T47D (23), contains one copy of the MMTV promoter and luciferase reporter integrated into the genome. This cell line was grown at 37 ºC with 5% CO_2 in RPMI 1640 medium containing 10% FBS, 1% glutamine, 1% penicillin/streptomycin, 100μl insulin and 700μg/ml G418 (Invitrogen). H1 variants knock down cell lines and HA-tagged H1 variant-expressing cell lines are derived from T47D-MTVL and in consequence, cultured in the same conditions.
- Cell lines H1 knocked-down MCF7 were derived from MCF7 cells from human mammary carcinoma. This cell line was grown at 37 ºC with 5% CO_2 in MEM medium containing 10% FBS, 1% penicillin/streptomycin, 1% aminoacids non-essential, 1% sodium pyruvate and 1% glutamine.
- Cell line H1 knocked-down MCF10A were derived from MCF10A cells, a non-tumoral epithelial mammary cell line. This cell line was grown at 37 ºC with 5% CO_2 in F-19 MEM medium containing 10% FBS, 1% penicillin/streptomycin, 100μl insulin and (…).
- Cell line H1 knocked-down 293T were derived from HEK 293T cells. Culture conditions were the same as the original cell line (described above).
- Cell lines H1 knocked-down HeLa were derived from Hela cells from human cervis carcinoma. This cell line was grown at 37 ºC with 5% CO_2 in DMEM medium containing 10% fetal bovine serum and 1% penicillin/streptomycin.

6. Virus production and infection for establishing stable cell lines.

293T and GP2-293 packaging cell lines (Clontech) were used for the production of viral particles.

GP2-293 packaging cell line (Clontech) is a cell line that expresses the viral *gag* and *pol* genes and was used for producing MoMuLV-derived virus. 2.5 x 10^6 cells were seeded in 100 mm-plates the day before transfection. To produce infectious particles, we co-transfected GP2-293 with our pSuper.Retro-shRNA vector and pVsVg, a plasmid that expresses VsVg envelope protein from the CMV promoter. It improves the spectrum of infection. Transfection was made using Lipofectamine Plus reagent (Invitrogen) according to its protocol. 48hr post-transfection, the culture medium was collected and used for infection of T47D-MTVL cells. Cells were infected by spinoculation method as follows: plates were centrifuged at 1,200 x g for 2 hr at 25 ºC and then, medium was replaced the day after. Infected cells were selected by puromycine resistant phenotype.

HEK 293T cell line (Clontech) was used to produce viral particles with the HIV-derived vectors tTR-KRAB-Red, and pLV-THM. 2.5x10^6 cells were seeded in p10 plates the day before transfection. Our vector was cotransfected with pVsVg and pCMVΔR8.91 (which expresses HIV *gag* and *pol*). Transfection was made using Calcium Phosphate (BD Bioscience) according to its protocol. 24 hours post-transfection, medium was collected and replaced with new medium. 24 hours later, medium was collected again, pooled and centrifuged 1h30min at 26.000 rpm at 4ºC in a sucrose gradient to concentrate viruses. Pellet containing viral particles was resuspended in 1ml of medium and used for cell infection. Cells were infected using spinoculation method as described above. The inducible knocked-down cell lines infected with ptTR-KRAB-Red and pLVTHM vectors were sorted for Red positive and GFP positive fluorescence after 3 days of doxicycline treatment. The HA-tagged H1 variant-expressing cell lines infected with pEV833 vector were sorted for GFP positive fluorescence.

7. Transient transfections.

24 hours before transfection, HEK 293T cells were seeded at a density of 2 x 10^6 cells in 100 mm-plates in DMEM medium. Transfection was made using Calcium Phosphate (BD Bioscience) according to its protocol. The calcium phosphate-DNA mix was prepared with 20 µg of DNA into 500µl of 250 mM $CaCl_2$. This mix was added to an equal volume of 2X HBS solution and incubated for 30 min at room temperature. Then, the suspension was added to the plates and eight hours later, the medium was replaced by fresh medium. Extracts were prepared 48h after transfection.

8. FACS analysis.

Samples for citometry analysis were fixed in 1X PBS : paraformaldehid 1% and analyzed using a FACS Calibur machine (Becton Dickinson) and CellQuest analysis software.

Propidium Iodide Staining Assays.

Cells were trypsinized, washed with PBS 1X and resuspended in PBS 1X-5 mM EDTA. Fixation was done adding ethanol to a final concentration of 70% and incubating the samples for 1h at -20ºC. Then cells were washed several times to get rid of ethanol and dissolved in Analysis solution.

Analysis Solution: 3% solution A, 3% solution B in PBS 1X.

Solution A: Ribonuclease A (Sigma) 10 mg/ml in PBS 1X.

Solution B: 38 mM sodium citrate, propidium iodide (PI) 500µg/ml in water.

9. Protein extracts.

Acid extraction of histone H1.

to the acid extraction method with 5% perchloric acid (1ml per $8x10^6$ cells, approximately) suggested by Banks et al (2001). After incubating the samples for 1 hour at 4ºC, they were centrifuged at maximum speed at 4ºC for 15 min. Trichloroacetic acid was added to the supernatant to a final concentration of 30% and inc Cells were washed twice with cold 1X PBS and lysed according ubated over night at 4ºC. After centrifuging at max speed for 15 min at 4ºC, the precipitated proteins were washed twice with 0.5 ml of acetone, dried for 5 min, reconstituted in 0.1 ml of water and stored at –20ºC.

Acid extraction of total histones.

Cells were scraped in cold PBS 1X. After the centrifugation of the samples (1200rpm at 4ºC), the pellet was resuspended in Lysis buffer A and homogenyzed with a pestle A, a mechanical procedure for breaking cells. Then, samples were washed with buffer B. 0.4 M of H_2SO_4 was added to the samples and incubated 1 hour on ice. After centrifuging 5 minutes at maximum speed, the supernatant was recovered and treated with NH_40H and ethanol over night at -20ºC. The pellet was washed two times with ethanol 50%, the dried pellet was resuspended in water and stored at -20ºC.

Buffer A: 10 mM Tris-HCl pH6.8, 50mM $Na_2S_2O_4$, 1% Triton, 10 mM $MgCl_2$, 8,6 g of sucrose in 100 ml and 0.2 mM PMSF.

Buffer B: 10 mM Tris-HCl pH7.5, 13mM EDTA and 0.2 mM PMSF.

Nuclear extract.

Cells were spun at 3000 rpm for 5 min, washed twice with cold 1X PBS. Pellet was resuspended in buffer A, swelled 10 minutes on rotator and homogenized with B pestle 10 times. After 10 minutes of centrifugation at 2500 rpm at 4ºC, the supernatant was removed (Cytoplasmic fraction). Pellet was dissolved in buffer B and homogeneized 10 times with pestle B. After 30 minutes in rotation at 4ºC, the cells were pull-down for 30 minutes at 12000 rpm. Supernatant (Nuclear Extract) was dialyzed in BC200 buffer for 4 h at 4ºC and stored at -80ºC.

Buffer A: 10mM Tris-HCl pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5M DTT and 0.2mM PMSF.

Buffer B: 20mM Tris-HCl pH 7.9, 25% glycerol, 1.5mM $MgCl_2$, 0.42M NaCl, 0.2mM EDTA, 0.5mM PMSF and 0.5mM DTT.

Buffer BC200: 25mM Tris-HCl pH7.9, 10% glycerol, 0.2mM EDTA, 200mM KCl and 0.2mM PMSF.

Whole cell extract.

Cells were washed twice with cold 1X PBS and scraped with Lysis Buffer. Samples were boiled 5 min to 95ºC and centrifuged at maximum speed. Supernatant was frozen at -20ºC.

Lysis Buffer: 25mM Tris-HCl pH7.5, 1% SDS, 1mM EDTA pH8.0, 1mM EGTA pH8.0, 20 mM β-glycerolphosphate, 2mM ortovanadate and 2mM PMSF.

10. Immunoprecipitation Assays.

Nuclear extracts from HEK 293T, HEK293F or T47D-MTVL were dialyzed in BC200 as it is described above and quantified by Bradford method.

For IPs against HA: 500 ug of total extract was incubated with anti-HA beads (Sigma) over night at 4ºC on rotation. Next day samples were washed 3 times with BC200 and eluted with a competitor peptide against HA (Sigma). Elution was repeated twice.

For IP against Ezh2: prot-A beads already blocked (Upstate) were incubated for 2 hour at 4ªC with the specific Ezh2 antibody in PBS 1X. After two washes with PBS 1X, 500 ug of nuclear extract was added and incubated at 4ªC over night. Next day samples were washed as it has been described before. Elution was done adding loading buffer and boiling the samples 5 minutes. Samples are ready for western-blot analysis.

11. SDS-PAGE and Western Blot analysis.

Extracts or purified proteins were run in 12% SDS-acrylamide gel and transferred to a nitrocellulose membrane. They were blocked for 1 hour at room temperature with 5% non-fat milk in TBS-T (1M Tris-HCl pH 7.4, 5M NaCl, 20X Tween). Membranes were incubated over

night at 4ºC with primary rabbit antibodies and then with secondary anti-rabbit antibodies for one hour at room temperature. Bands were visualized by chemiluminiscence using the ECL system (Amersham).

12. FPLC purification and fractionation of histone H1 subtypes.

The samples obtained after H1 purification procedure were further purified in a FPLC system using an Amersham Resource RPC 1ml column with a linear gradient from 41% to 44.5% buffer B. Buffer A consisted of 0.065% trifluoroacetic acid (TFA) and 2% acetonitrile acid (ACN) in water. Buffer B consisted of 80% ACN, 0.05% TFA plus water. The elution profile was constructed from absorbance measurements at 210 nm. Purified samples were dried in a speed-vac, dissolved in water, and stored at –20ºC.

13. Electrospray mass spectrometry.

H1-purified extracts for mass spectrometry were prepared as described above. The samples were processed in the Proteomics Facility at Pompeu Fabra University for mass determination between 20,500 and 23,100 Da.

14. RNA extraction and RT-PCR.

Total RNA was prepared by using the RNAsy Kit (Qiagen) as described in the manufacturer's instructions. The RNA obtained is dissolved in RNase-free water and stored at -80ºC. The quality of the RNA was analyzed using the Agilent Bioanalyzer 2100® and the RNA 6000 LabChip Kit (Agilent®) with the Eukaryote Total RNA Nano Assay®.

The cDNA was generated from 100 ng of total RNA by using Superscript First Strand Synthesis System (Invitrogen). Gene products were analyzed by qReal-Time PCR using SYBR Green (Roche) and specific oligonucleotides. Each value was corrected by human GAPDH and expressed as relative units. Primer sequences are summarized in point 2.

RNA for microarray experiments.

Cells were seeded at a confluency of 60% in white RPMI 1640 medium containing 10% of charcolized serum and 1% penicillin/streptomycin. 36 hours later, media was replaced by fresh media without serum. After one day in serum-free conditions, vehicles/R5020 (10nM) were added to the media. After 6h of incubation, cells were harvested and total RNA was isolated using RNAsy Kit (Qiagen). To check the quality of the RNA, samples were analyzed using the

Agilent Bioanalyzer 2100® and the RNA 6000 LabChip Kit (Agilent®) with the Eukaryote Total RNA Nano Assay®.

In the case of the inducible shRNA expression system, cells were pretreated with doxicycline (2.5μg/ul) for 3 days before seeding the cells for the experiment. During the experiment described above new doxicycline has been added every time the media has been changed. At the end, the induction of the shRNA took six days. The rest of the protocol is as described above.

15. Microarray and data analysis.

Samples were analyzed by the Microarray Unit at the Centre de Regulació Genòmica (CRG).

Raw data was processed using MARGE, an in house developed web implementation of LIMMA, a microarray statistical analysis package of Bioconductor (http://www.bioconductor.org) that is run in the R programming environment. Discriminant factor analysis was run using an application of the software FADA. Gene intensities were background subtracted (taking mean of channel intensities and median of background). Spots with intensities <2 times the local background in either or both dye filter channels (Cy3 or Cy5) as well as controls were excluded from normalization, and were referred as “not reliable”. An intensity dependent normalization algorithm (global lowess) was applied using a smoothing factor f=0.2 for all experiments. Normalized Log_2Ratios (Intensity Cy5/Intensity Cy3) were scaled so that they all had the same median absolute standard deviation across all the arrays, to give the same weight to each gene, and not only due to the magnitude of the expression ratio. The computed B statistic rank value from all replicate hybridizations was used to determine the genes with significant changes. We considered genes that showed a 1.4-fold gene up or down-regulation relative to control sample with a B-rank value above the 90^{th} percentile as significant. The value of fold change or copy number relative change was calculated as $2^{Log2Ratio}$, if the value of the ratio was >0, or $2^{-1/Log2Ratio}$, if it was <0.

In order to do the statistical analysis of the data, we have used the open-source, freely available software package for microarray data management and analysis TM4 obtained from TIGR (http://www.tigr.org/software/).

16. PCR Array System (Superarray).

The PCR Array is a 96-well plate containing primers for a researched set of 84 relevant, pathway- or disease-focused genes, plus five housekeeping genes and three RNA and PCR quality controls. The system also includes an instrument-specific master mix and an optimized first strand synthesis kit. The PCR Array System combines the quantitative performance of SYBR® Green-based real-time PCR with the multiple gene profiling capabilities of a microarray. The platform used (APHS-020) is focused on cell cycle related genes.

Functional Gene Groupings

G1 phase and G1/S transition: ANAPC2, CCND1, CCNE1, CDC34, CDK4, CDK6, CDKN1B, CDKN3, CUL1, CUL2, CUL3, SKP2.

S phase and DNA replication: ABL1, MCM2, MCM3, MCM4, MCM5, PCNA, RPA3, SUMO1, UBE1.

G2 phase and G2/M transition: ANAPC2, ANAPC4, DIRAS3, BCCIP, BIRC5, CCNB1, CCNG1, CCNH, CCNT1, CCNT2, CDK5R1, CDK5RAP1, CDK7, CDKN3, CKS1B, CKS2, DDX11, DNM2, GTF2H1, GTSE1, HERC5, KPNA2, MNAT1, SERTAD1.

M phase: CCNB2, CCNF, CDC2, CDC16, CDC20, MRE11A, RAD51.

Cell cycle checkpoint and cell cycle arrest: ATM, ATR, BRCA1, BRCA2, CCNG2, CDC2, CDC34, CDK2, CDKN1A, CDKN1B, CDKN2A, CDKN2B, CDKN3, CHEK1, CHEK2, CUL1, CUL2, CUL3, GADD45A, HUS1, KNTC1, MAD2L1, MAD2L2, NBN, RAD1, RAD17, RAD9A, RB1, RBBP8, TP53.

Regulation of cell cycle: ABL1, ANAPC2, ANAPC4, DIRAS3, ATM, ATR, BCCIP, BCL2, BRCA2, CCNB1, CCNB2, CCNC, CCND1, CCND2, CCNE1, CCNF, CCNH, CCNT1, CCNT2, CDC16, CDC2, CDC20, CDK2, CDK4, CDK5R1, CDK6, CDK7, CDK8, CDKN1A, CDKN1B, CKS1B, DDX11, E2F4, GADD45A, KNTC1, MKI67, PCNA, RAD9A, RB1, SKP2, TFDP1, TFDP2.

Negative regulation of cell cycle: ATM, BAX, BRCA1, CDKN2B, RBL1, RBL2, TP53.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Gene 1	Gene 2	Gene 3	Gene 4	Gene 5	Gene 6	Gene 7	Gene 8	Gene 9	Gene 10	Gene 11	Gene 12
B	Gene 13	Gene 14	Gene 15	Gene 16	Gene 17	Gene 18	Gene 19	Gene 20	Gene 21	Gene 22	Gene 23	Gene 24
C	Gene 25	Gene 26	Gene 27	Gene 28	Gene 29	Gene 30	Gene 31	Gene 32	Gene 33	Gene 34	Gene 35	Gene 36
D	Gene 37	Gene 38	Gene 39	Gene 40	Gene 41	Gene 42	Gene 43	Gene 44	Gene 45	Gene 46	Gene 47	Gene 48
E	Gene 49	Gene 50	Gene 51	Gene 52	Gene 53	Gene 54	Gene 55	Gene 56	Gene 57	Gene 58	Gene 59	Gene 60
F	Gene 61	Gene 62	Gene 63	Gene 64	Gene 65	Gene 66	Gene 67	Gene 68	Gene 69	Gene 70	Gene 71	Gene 72
G	Gene 73	Gene 74	Gene 75	Gene 76	Gene 77	Gene 78	Gene 79	Gene 80	Gene 81	Gene 82	Gene 83	Gene 84
H	HK1	HK2	HK3	HK4	HK5	GDC	RTC	RTC	RTC	PPC	PPC	PPC

17. ChIP protocol.

ChIP assays were performed as described (Strutt, Paro 1999) by using chromatin from T47D-MTVL cells expressing HA-tagged H1 variants.

The cells were treated with R5020 10 nM for the appropriate time. After R5020 treatment, medium was replaced with fresh medium (serum-free medium without phenol red) and proteins were crosslinked to DNA by adding Crosslinking solution, containing formaldehyde, directly to culture medium to a final concentration of 1% formaldehyde, and incubating for 10

min at 37ºC. Then the crosslinking reaction was stopped by adding Glycine to a final concentration of 0.1M and incubating for 5 min at room temperature. The medium was removed and the cells were washed twice using ice cold PBS containing protease and phosphatase inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1μg/ml aprotinin, 1μg/ml pepstatin A, 1μM sodium ortovanadate, 20 mM β-Glycerophosphate and 1X Protease inhibitors cocktail (Roche)). The cells were scrapped in PBS containing inhibitors and pelleted for 5 min at 4000 rpm at 4ºC. Cell pellets were resuspended in 2.5 ml of Lysis Buffer containing inhibitors and incubating for 10 min on ice. After lysis, cells were pelleted for 5 min at 4000 rpm at 4ºC and then resuspended in 1 ml of Nuclei Lysis Buffer.

Lysate was sonicated on ice using Biorruptor machine to shear DNA to lengths between 300 and 200 bp. Sonicated matherial was centrifugated 5 min at 4000 rpm at 4ºC, to recover the supernatant (chromatin) and discard the cell debris. An aliquot of the chromatin was treated with Proteinase K and the DNA was recovered by phenol / chloroform extraction. DNA was quantified by Nanodrop and 300 ng of DNA were run in a 1.2% agarose gel to confirm that the size of the sheared DNA was 250-300 bp.

To perform the chromatin immunoprecipitation, 20-30 ug per sample of quantified chromatin was diluted 10-fold in ChIP Buffer. To reduce non-specific background, the diluted chromatin was pre-cleared with 15 ul of Salmon Sperm DNA / Protein A or G Agarose – 50% Slurry (Upstate) for 4 h at 4ºC with rotation. The pre-cleared chromatin was collected by brief centrifugation and the corresponding immunoprecipitating antibody was added to the supernatant fraction (1ug for PR antibody, 3ug for HA antibody), and was incubated over night at 4ºC with rotation. Control for non-specific interaction of DNA was performed by using as non-specific rabbit IgG antibody (Sigma). For input control, an aliquot of the pre-cleared chromatin was recovered before antibody incubation. 30 ul of Salmon Sperm DNA / Protein A– 50% Slurry were added for 2 h at 4ºC with rotation, to collect the antibody/proteins/DNA complexes. The agarose was pelleted by gentle centrifugation (1 min at 3600 rpm at 4ºC) and supernatant containing unbound inespecific DNA was discarded. The agarose with bound antibody/protein/DNA complexes was washed for 5 min at 4ºC with rotation with each of the Washing Buffer 1, 2 and 3, and then twice with TE1X. The DNA was eluted by incubating twice the washed agarose with Elution Buffer for 15 min at room temperature with rotation. The supernatant was recovered by centrifugation for 5 min at 3600 rpm at room temperature. 0.2M NaCl was added and samples were incubated overnight at 65ºC to reverse crosslinking, followed by treatment with proteinase K and DNA recovered by phenol / chloroform extraction. DNA was precipitated with 100% ethanol, 10% sodium acetate and 0.1% glycogen, washed with 70% ethanol, and finally, the DNA pellet was dissolved in DNase-free water.

For each experiment, PCRs were performed with dilutions of input DNA to determine the linear range of amplification. The human □-globin gene was used as a control. Primer sequences were previously summarized.

ChIP Solutions:

Crosslinking Solution: 50mM Hepes pH8.0; 0.1M NaCl; 1mM EDTA pH8.0; 0.5mM EGTA pH8.0.

Cell Lysis Buffer*:* 5mM Pipes pH8.0; 85mM KCl; 0.5% NP-40

Nuclei Lysis Buffer*:* 1% SDS; 10mM EDTA pH8.0; 50mM Tris-HCl pH8.1

ChIP Buffer: 0.01% SDS, 1.1% Triton X-100; 1.2 mM EDTA pH8.0; 16.7 mM Tris-HCl pH8.1; 167mM NaCl

Washing Buffer 1: 0.1% SDS; 1% Triton-X100; 2mM EDTA pH8.0; 20mM Tris-HCl pH8.1; 150mM NaCl

Washing Buffer 2: 0.1% SDS; 1% Triton-X100; 2mM EDTA pH8.0; 20mM Tris-HCl pH8.1; 500mM NaCl

Washing Buffer 3*:* 0.25M LiCl; 1% NP-40; 1% Sodium Deoxicholate; 1mM EDTA pH8.0; 10mM Tris-HCl pH8.1

Elution Buffer: 1% SDS, 0.1M $NaHCO_3$

18. Tiling array for ChIP-on-chip experiments using Agilent Technologies.

A custom tiling array was designed by the group covering regions from -8.0 Kb upstream to +2.0 Kb downstream of the transcriptional start sites of 42 selected genes. These genes are progesterone responsive genes identified by oligonucleotide microarrays (Agilent Technologies: Human 1A Oligo Microarray (V2) containing 22,000 unique 60-mer oligonucleotides). The sequences were tiled with 60 mer in situ-synthesized oligonucleotides with 20 bp periodicity (40 bp overlap), generating an average of 497 probes per gene. Probes with high Tm's were trimmed one base pair at a time until either the probes reaches 45 bp in length or a Tm < 80.0 degrees.

19. Inmunofluorescene.

Cells were seeded onto coverslips in rich medium. 36h later, cells were washed, fixed by incubation in 4% paraformaldehyde in PBS 1X for 15 minutes at room temperature, and permeabilized by incubation in 0.2% triton X-100 in PBS for 10 min at room temperature. After rinsing three times for 5 min in PBS, the coverslips were incubated for 1h with 3% BSA in PBS at RT to reduce non-specific staining. Cells were incubated with specific antibodies in 3% BSA in

PBS for another hour at RT. After several washes in PBS, coverslips were exposed to biotinilated-secondary antibody antimouse or rabbit (depending on the antibody) diluted 1:200 in 3% BSA in PBS for 1h at RT. Then coverslips were washed in PBS and mounted on slides with VectaShield – DAPI mounting medium (Vector Laboratories) and subjected to Leica DM IRBE inverted research microscope.

RESULTS

1. Generation of tools for studying the role of H1 variants in cell processes.

1.1- Production of isoform-specific antibodies for the linker histone H1.

1.1.1- Production of specific antibodies against somatic H1 subtypes.

Due to the lack of specific commercial available antibodies against H1 subtypes, we decided to first generate our specific polyclonal antibodies against each somatic variant (from H1.1 to H1.5). Specific N-terminal peptides for each protein were synthesized and used for immunizing rabbits (Figure 1A). The serums extracted were purified by protein-A affinity column. All the antibodies recognized specifically their peptides (Figure 1B) as well as their recombinant variants (Figure 1C). The only exception is the H1.4 antibody that, despite recognizing its immunizing peptide, does not react against the full-length H1.4 protein. Importantly, H1.2, H1.3 and H1.5 antibodies do not cross-react with other members of the family in H1 extractions from T47D cells (Figure 1D) and H1.1 variant is not present in our cell model. We have also performed immunoprecipitation assays to test if our antibodies were good enough to use in ChIP experiments but our results (data not shown) indicate that they were not able to immunoprecipitate H1 proteins. These antibodies have been used extensively for western-blot in our studies.

Moreover, taking advantage of the recombinant H1 variants (courtesy of Dr. Nicole Happel) we performed a screening of several commercial available antibodies. The pattern of recognition of the indicated antibodies is shown in Figure 2. The most remarkable thing is that phospho-H1 antibody from Upstate as well as p-T146 from Abcam recognized the subtype H1.4. Both antibodies have been used as an indirect H1.4 detection method.

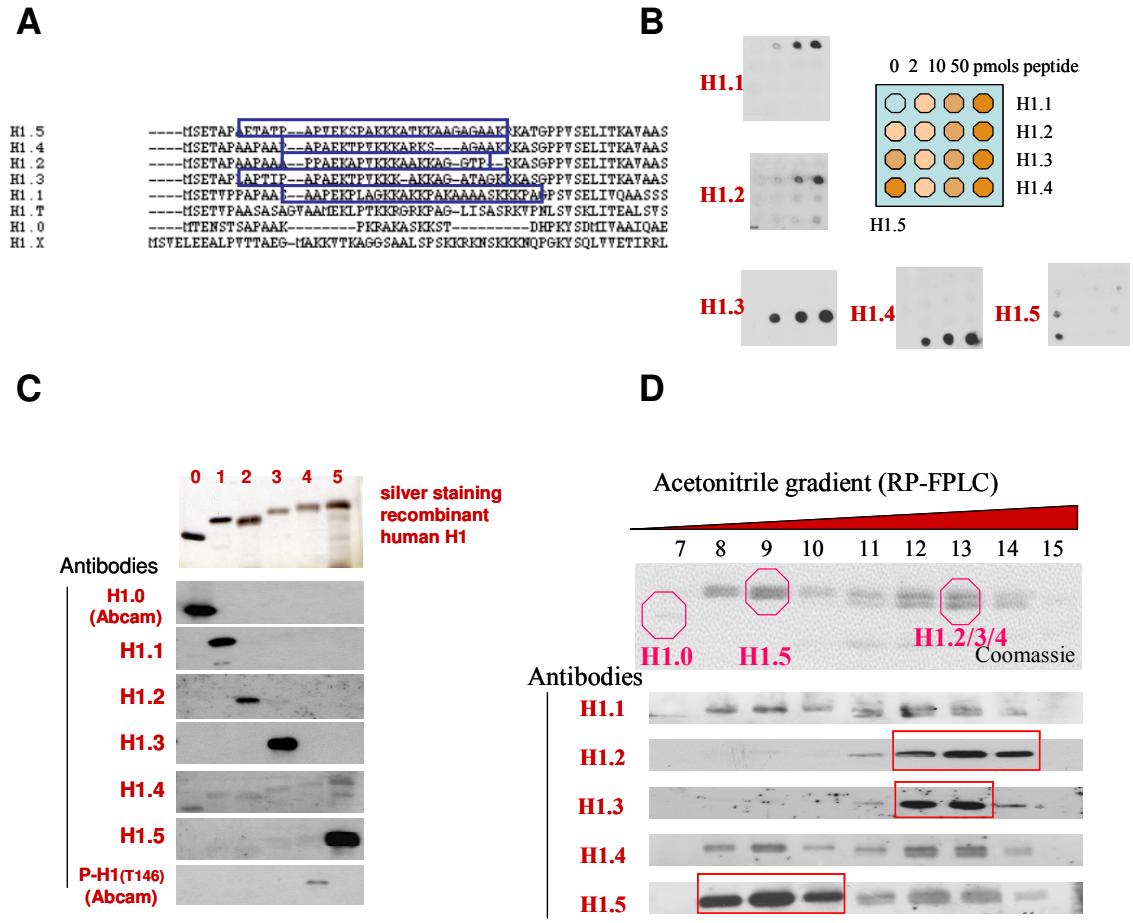


Figure 1. **Production of new human histone H1 variant-specific antibodies.**

(A) Alignment of N-terminal sequences of human H1 variants underlying peptide sequences used for production of polyclonal antibodies.

(B) Immunodot-blot of H1 variant antibodies against their specific peptides.

(C) Western blot analysis of H1 variant antibodies against recombinant human H1 variants produced in yeast. H1.1, 2, 3 and 5 recognize specifically its corresponding variant. Sample (ch) corresponds to a chromatin preparation from a breast cancer cell line (T47D).

(D) Separation of H1 variants by reverse phase-FPLC and recognition by specific antibodies. H1 variants extracted from T47D cells were resolved in an acetonitrile gradient and submitted to western-blot with antibodies generated in-house.

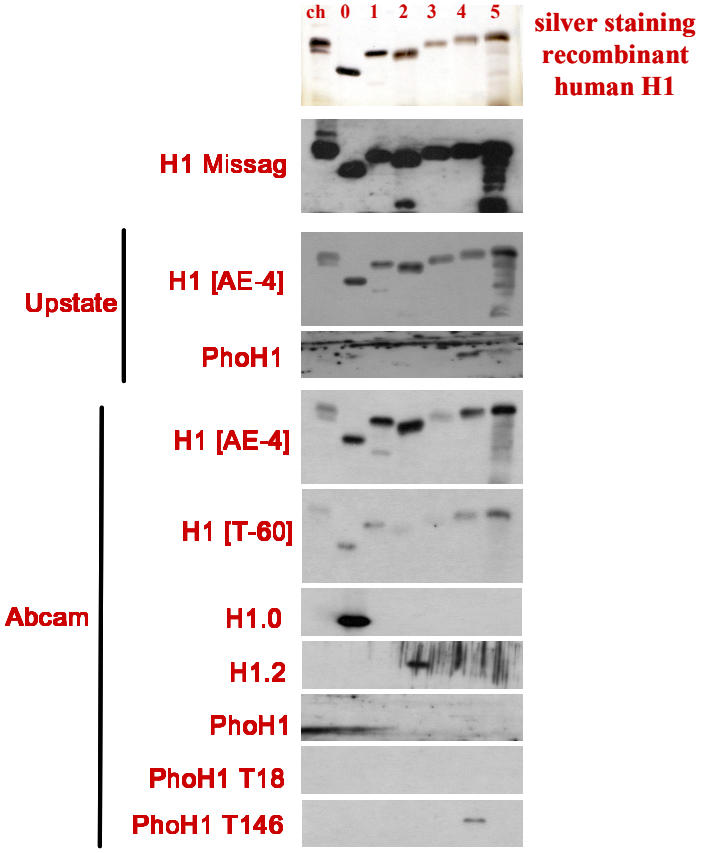


Figure 2. Characterization of other existing commercial H1 antibodies. Extending western-blot analysis to other existing antibodies we established that the commercial H1.0 antibody (Abcam) is highly specific, and commercial Pho-H1 antibodies (for instance T146) recognize H1.4 variant.

In order to improve the quality and the antigenicity of the H1 isoform-specific antibodies we started a collaboration project with Abcam Ltd. (Cambridge, UK). Following our instructions, they produced antibodies against the non modified proteins by using a two branched-peptide that encompasses the divergent selected region of each variant by five amino acids on either side (Perez-Burgos et al 2004). This novel system described by Dr. Thomas Jenuwein's group permits to obtain antiserums with both higher titer and higher specificity. Many serums for H1 variants (from H1.2 to H1.5) have been tested but only specific antibodies against H1.2, H1.3 and H1.5 were obtained (Figure 3). At this moment these antibodies are being commercialized by Abcam. Unfortunately, their applications are restricted for western-blot techniques as far as we have tested.

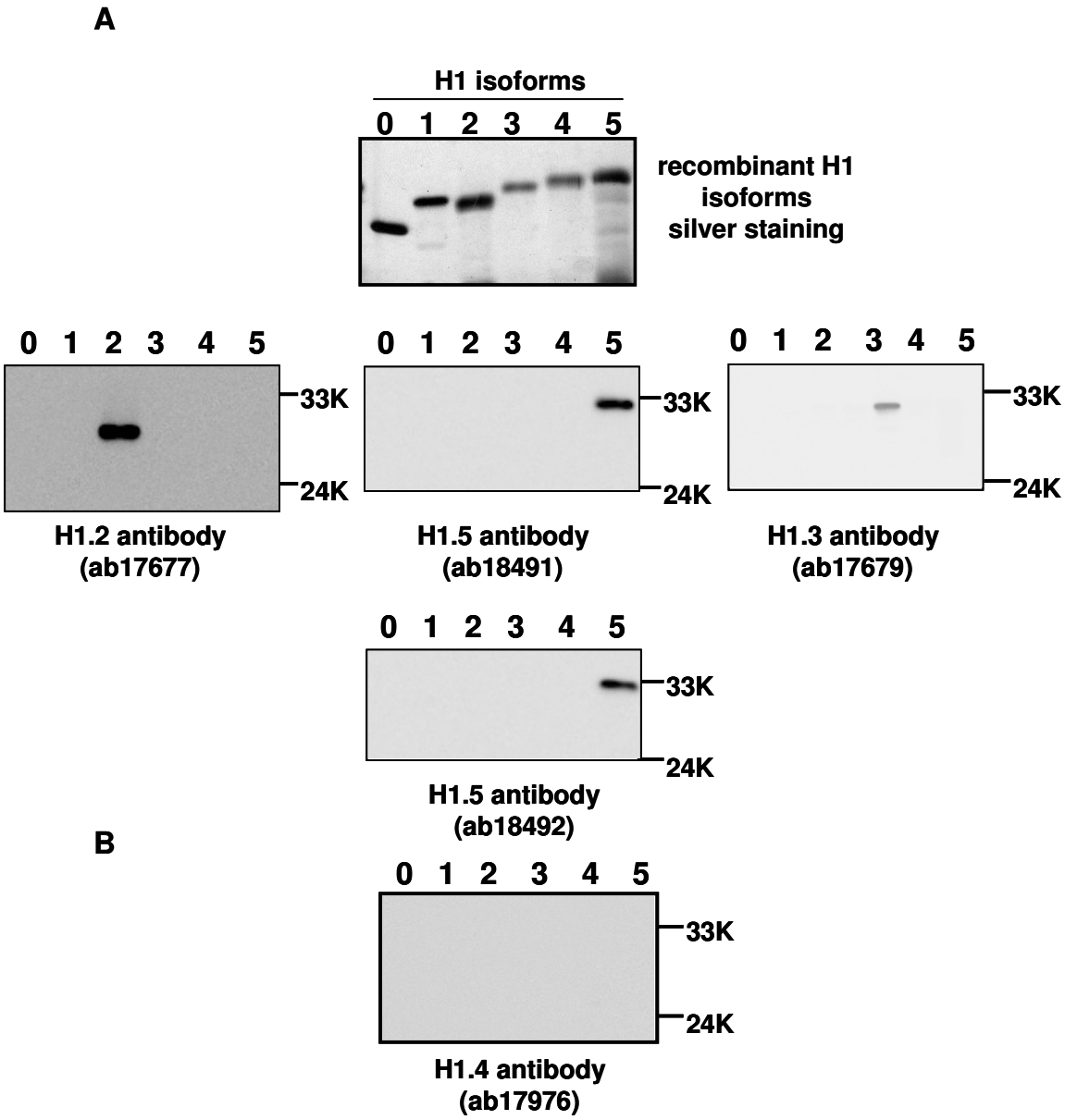


Figure 3. Analysis of the specificity of the Abcam-produced antibodies.

(A) Commercial antibodies against H1.2, 3 and 5 recognize specifically its corresponding variant in a Western-blot with recombinant human H1 variants.

(B) H1.4 antibody does not recognize any histone variant.

Nowadays, the collaboration project with Abcam is focused on the development and characterization of rabbit polyclonal antibodies that are directed against modified positions in the different H1subtypes.

1.1.2- Production of antibodies against post-translationally modified H1 variants.

Histones play, in addition to their structural roles, important functions in the control of gene expression by regulating access to the underlying nucleosomal template. Histone tails are subject to a variety of post-translational modifications as it has been described in the Introduction. Core histone tail modifications have been proposed to constitute a "histone code" that could significantly extend the information potential of the genetic information (Strahl and Allis 2000; Turner 2000; Jenuwein and Allis 2001). Based on these implications, modified core histone antibodies have been extensively developed. However, the lack of specific antibodies for H1 subtypes and the controversial role of H1 as transcriptional regulator have limited the research in this area.

In the past years, several publications showing specific roles for H1 subtypes, their involvement in gene expression and the identification of some post-translationally modified residues have increased the interest in the development of tools for the study of the role of H1 variants in transcription. For this purpose, we are generating together with Abcam, new antibodies against modified residues in different H1 variants (Table 1). These modifications have been described by proteomics approaches (Sarg et al. 2005; Wisniewski et al. 2007). The characterization of the first arriving antibodies has already started.

branched peptides: **2x(……..)-1xLys-Cys**

H1 isoform	**modific.**	**residue**	**sequence**	
4	Pho/Pho	S2/T4	**ac-pS**E**pT**APAAPAA	
4	unmodif.		VKKKAR**KS**AGAA	
4	Met	K26	VKKKAR**meK**SAGAA	mono, di, **tri** Methyl
4	Ac	K26	VKKKAR**ac**KSAGAA	
4	Pho	S27	VKKKARK**pS**AGAA	
4	Met/Pho	K26/S27	VKKKAR**meKpS**AGAA	mono, di, **tri** Methyl
4	Ac/Pho	K26/S27	VKKKAR**acKpS**AGAA	
2,3,4	Pho	S36	RKA**pS**GPPVSELIT	
4	Pho	S36	AGAAKRKA**pS**GPPV	
2	Met	K26	KAA**meK**KAGG**T**PR	mono, di, **tri** Methyl
2	Ac	K26	KAA**ac**KKAGG**T**PR	
2	Pho	T31	KAA**K**KAGG**pT**PR	
2,3,4	Ac	K63	VSLAAL**acK**KALAAA	

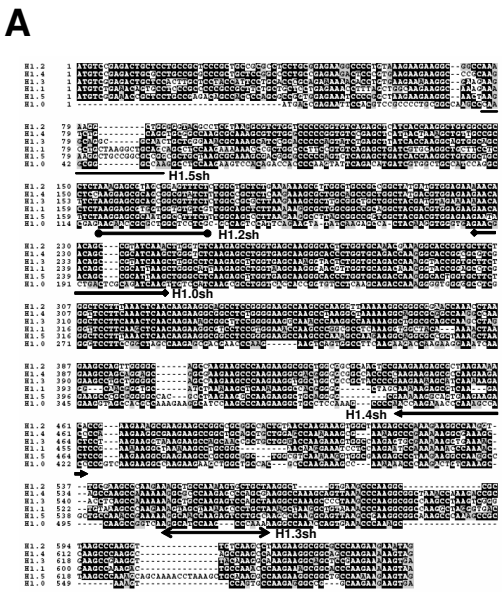
Table 1. Proposed antibodies against modified H1 variants for Abcam.

Pho means phosphorylation of Serine (S) or Treonine (T). Met is methylation of Lysine (K) and Ac acetylation of Lysine (K).

1.2- Generation of stable shRNA mediated knocked-down cell lines for histone H1 variants in human breast cancer cells.

We next generated stable breast cancer cell lines lacking expression of each of the H1 variants specifically in order to investigate the role of each variant on global gene expression and cell proliferation. For that purpose, we set up both H1 shRNA stable and inducible expression systems in T47D-MTVL, a breast cancer cell line with a copy of the MMTV promoter, a model promoter for steroid hormone induction, integrated into the genome.

The shRNAs selected to inhibit H1.0, H1.2, H1.3, H1.4 and H1.5 proteins encode two 19 nucleotide reverse complements homologous to the most divergent regions of the different target genes in order to be specific for each H1 isoform (Figure 4A). These oligonucleotides are separated by a short spacer region that forms a hairpin responsible for activating Dicer machinery, RISC complex and finally, target mRNA degradation. Up to 25 different shRNAs have been tested and only one per each H1 variant was selected for inhibition (Figure 4B).



B

	Sequence position	Target Sequence
H1.0	54	GGCCTCCAAGAAGTCCACA
	104	CCATCCAGGCCGAGAAGAA
	190	CGCTGACTCGCAGATCAAG
	290	GAGCGACGAACCCAAGAAG
H1.2	51	GGCCCCTGTAAAGAAGAAG
	156	AGAGCGTAGCGGAGTTTCT
	379	ACCTAAGAAGCCAGTTGGG
	436	CTCCGAAGAAGAGCGCTAA
H1.3	73	GAAGGCAGGCGCAACTGCT
	88	CTGCTGGGAAACGCAAAGC
	140	GGCAGTGGCAGCTTCTAAG
	473	GGTAAAGAAGCCAGCAACC
	491	CCGCTGCTGGGACCAAGAA
	506	AAAGTGGCCAAGAGTGCGT
	555	CTGCCAAGAGTCCAGCTAA
H1.4	120	GTCCGAGCTCATTACTAAA
	225	GAACAACAGCCGCATCAAG
	444	GAAGAGCGCCAAGAAGACC
	458	GACCCCAAAGAAGGCGAAG
H1.5	75	GGCAACTAAGAAGGCTGCC
	165	GGAGCGCAATGGCCTTTCT
	371	GAAGGCAGGCGCCGCTAAA
	419	GAAGGCCAAGAAGGCTGCA
	562	AAGAGTCCTGCCAAGCCCA

Figure 4. Selected target sequences for shRNA-mediated inhibition.

(A) Alignment of human histone H1 variants-encoding genes showing the sequences targeted by the different shRNAs used in this report.

(B) List of all the oligonucleotides for shRNA tested. Working shRNAs are indicated in red.

1.2.1- Constitutively H1 variant knocked-down T47D-MTVL cell lines.

All the shRNAs designed were cloned into the pSuper.Retro vector for a constitutive expression (Figure 5A). This vector has a retroviral backbone that permits to introduce the shRNAs in T47D-MTVL cell line by infection with viral particles (Figure 5B). We selected populations for puromycin resistance and few clones were generated.

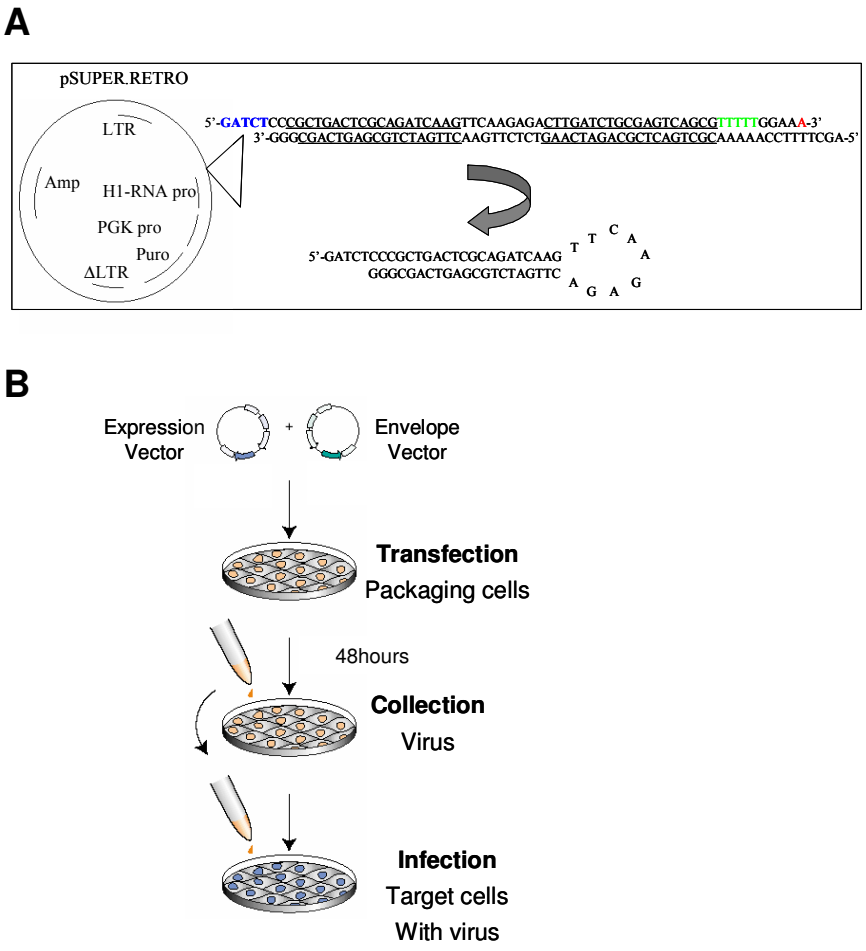


Figure 5. Constitutive shRNA expression system.

(A) Schematic drawing of the pSuper.Retro vector. The main characteristics of the vector are indicated. One example of dsDNA oligonucleotide for cloning is represented, as well as the predicted shRNA transcript. This secondary structure is the optimal substrate for Dicer enzyme that will generate the inhibitory RNAi.

(B) Graphic representation of the generation of viral particles and the consecutive infection.

In all cases the inhibition of H1 subtypes was analyzed by western-blot using our home-made antibodies and H1.0 antibody and anti-phospho T146-H1 antibody, which recognizes also H1.4, both from Abcam (Figure 2).

Western blotting analysis showed one working shRNA for each variant: H1.0 190sh, H1.2 156sh, H1.3 555sh, H1.4 444sh and H1.5 75sh. As the inhibition was not complete and the behavior of the cell population was not homogenous we generated clones from these pools of cells (Figure 6A).

While the inhibition of H1.0 in the majority of the clones was very stable and reproducible, in the case of variants H1.2, H1.3 and H1.5 many of the clones were not inhibiting. Moreover, we observed that the inhibition of the clones was not stable and maintained along time (data not shown) for the majority of the H1 subtypes. In addition, most of the knocked-down H1.4 clones died after some weeks in culture. Western blotting experiments with our best cell lines for the inhibition and all the collection of antibodies showed no significant changes in the proportion of other variants (Figure 6B), except a plausible increase of H1.3 in the knocked-down H1.5 cell line.

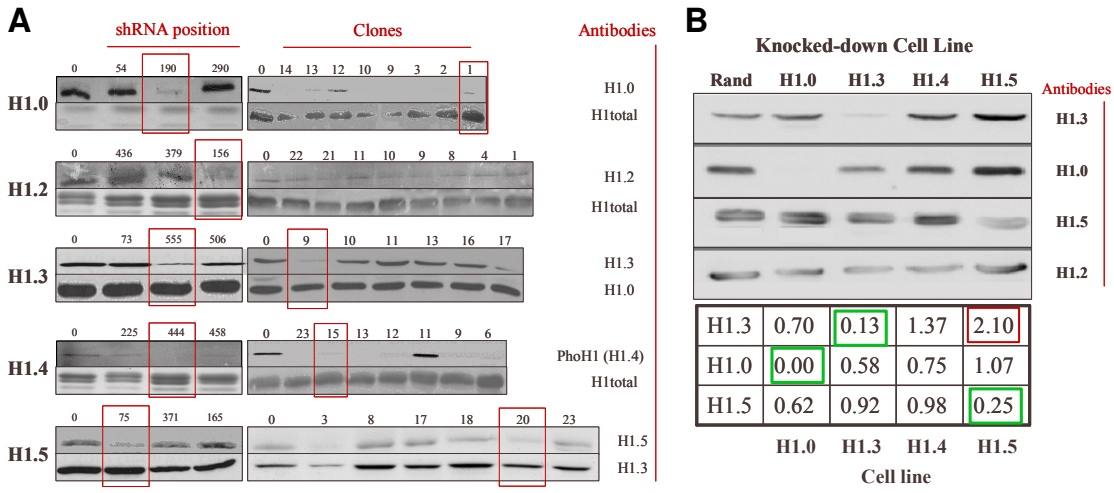


Figura 6. Characterization of H1 variant inhibition by constitutively-expressed shRNAs in T47D.

(A) Detection of H1 shRNA-mediated isoform inhibition. Western blot analysis of H1 purified from T47D-MTVL cells infected with different constitutive shRNA expression vectors. The left panel shows the western blot of the pools of cells infected with different shRNAs and the right panel the individual clones generated from the pool of cells where a particular shRNA was working. Total H1 antibody (Abcam) or another variant antibody was used as loading control.

(B) Pattern of expression of all H1 isoforms in each knocked-down cell line. Western blot of purified H1 from constitutively knocked-down H1.0 to H1.5 T47D-MTVL cell lines and the control cell line (rand) expressing a random shRNA with all available antibodies. The lower panel shows a relative

quantification of western bands, were each band intensity has been corrected by H1.2 as loading control and the amount of H1 variants in the random shRNA cell line. Green squares mark specific inhibitions, red squares denote hypothetical compensatory effects.

Data indicates that it was not possible to keep for long time the knocked-down H1 clones, suggesting the presence of deleterious effects triggering the selection of those clones more resistant to the inhibition. Due to these inconvenients with the constitutive expression system, we decided to move to an inducible lentiviral shRNA expression system. The inducible system permits more accurate inhibitions during short times avoiding culture conditions for the cells that could lead to a possible adaptative process due to the lost of a specific H1 variant.

1.2.2- Conditional suppression of histone H1 subtypes in T47D-MTVL cell lines.

In order to conditionally suppress the expression of each H1 subtype, we have used a lentivirus vector-based system for drug-inducible production of shRNAs in stably transduced mammalian cells. The inducible expression system consists of a regulator vector (Lv-tTR-KRAB-Red) and the pLVTHM vector, that codifies for expression of the cloned shRNA and the reporter GFP (Wiznerowicz and Trono 2003). The ptTR-KRAB vector produces the tetracycline repressor tTR fused to the KRAB domain of human Kox1 (Deuschle et al. 1995). When linked to DNA-binding domain of tTR, KRAB can modulate transcription from an integrated promoter containing the tetO sequences. The regulated shRNA vector (pLVTHM) is under the control of a tetO-H1 promoter. In the absence of Dox, tTR-KRAB binds specifically to *tetO* and suppresses the activity of the nearby promoter. Conversely, in the presence of Dox, tTR-KRAB is sequestered away from *tetO*, thus permitting shRNA expression (Figure 7A).

To generate the inducible knocked-down H1 cell lines, we previously infected our T47D-MTVL cell line with the regulator vector (Lv-tTR KRAB-Red). Once we sorted the cells for Red positive fluorescence we did clones and selected one for the next future experiments. One of such cell lines, named T47D-MTVL-KRAB was infected with pLVTHM- shRNA vectors and sorted for both fluorescences (RedFP and GFP) after doxicycline addition (Figure 7B).

Once we have generated the inducible knocked-down cell lines for H1.0, H1.2, H1.3, H1.4 and H1.5 variants, we detected optimal inhibition at the protein level after 6 days of doxicycline treatment (Figure 7C) and at the mRNA level, after 2 days of treatment (Figure 7D), earlier than the apparent reduction of H1 proteins, as shown here for H1.2 as an example. Complete analysis of all H1 variants knock-downs is shown ahead (Figure 10).

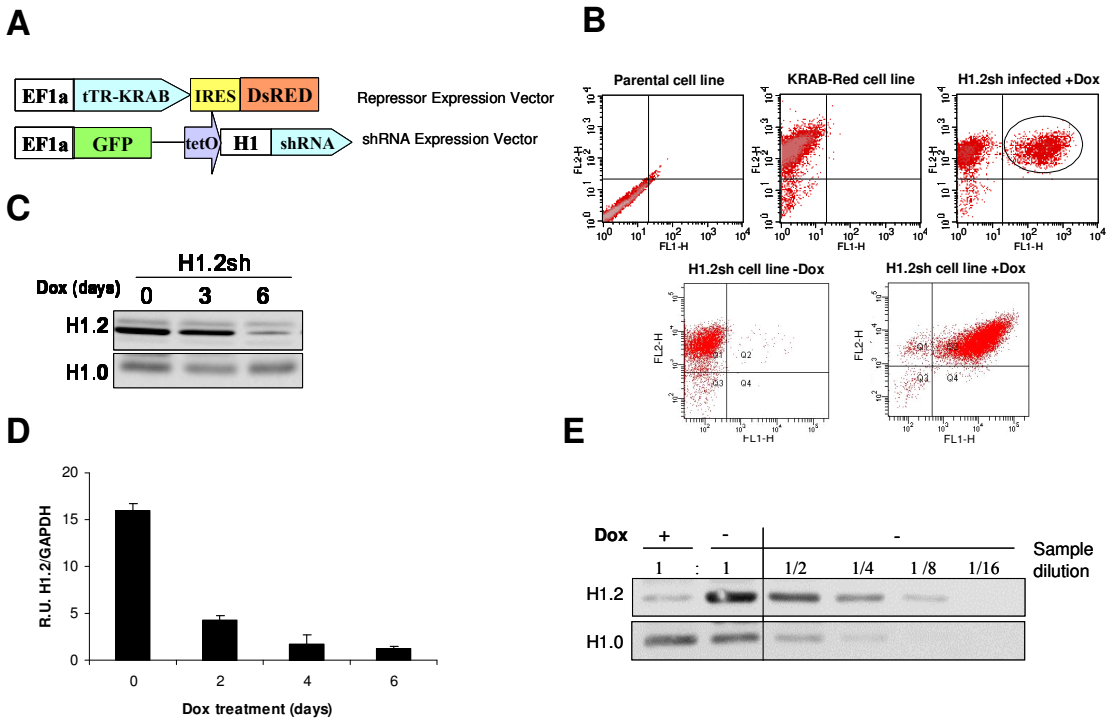


Figure 7. Inducible RNA interference-mediated knock-down of histone H1 variants in T47D.

(A) Scheme of the inducible, lentivirus-based shRNA-expression system used (Wiznerowicz et al. 2003). Cells are first infected with the pLV-tTR/KRAB-RedFP and FACS-selected for RedFP expression. Later, infected with the shRNA-expression vector pLV-THM. Only upon Dox addition, cells become GFP-positive and express the cloned shRNA.

(B) FACS analysis of cells infected with the shRNA-expression inducible system at the different stages of cell line construction. The example shown refers to H1.2 shRNA expression in T47D cells. GFP was measured in the FL1-H axis, RedFP in FL2-H. Cells sorted for GFP expression upon Dox treatment were kept in culture without Dox (GFP-negative). Upon Dox addition, all the population became GFP-positive and the effect of shRNA expression was analyzed.

(C) Western blot analysis of H1.2 depletion in a Dox treatment time-curve.

(D) Example of H1.2 mRNA reduction along a Dox time-course.

(E) Relative quantification of H1.2 depletion upon Dox treatment. Serial dilutions of the H1 sample extracted from non-treated cells were compared to a Dox-treated sample in a Western blot using anti H1.2 antibody. This comparison shows that H1.2 was reduced 8-fold. H1.0 antibody was used as loading control.

1.3- Generation of HA tagged H1 variant vectors.

The generation of HA tagged H1 variants was a tool developed in collaboration to Dr. Reinberg's laboratory. These tools allow us to explore in more detail which is the specific participation of the variants in cellular processes. Through the expression of the variant of interest we are able to immunoprecipitate specifically the protein by using commercial antibodies capable of recognizing the HA epitope.

The ORF of H1.0, H1.2, H1.3, H1.4 and H1.5 was amplified with specific primers from genomic DNA and then cloned in HA-containing $pCDNA_4$ vector (Figure 8A). This plasmid corresponds to a commercial vector for obtaining high levels of expressed-protein. The purpose was to study interactions of a particular H1 variant with other protein complexes by transient transfection in a cell model easy to transfect or to work such as 293F or 293T.

In addition to this, each of the H1 variants fused to a C-terminal HA tag from $pCDNA_4$ was cloned to the pEV833 vector, a lentiviral HIV-derived vector that permits to make viral particles and infect cells in order to make stable cell lines (Figure 8B). Stably expressing H1-HA variant cell lines are a powerful tool for studying the specific recruitment of one particular variant in a promoter of interest.

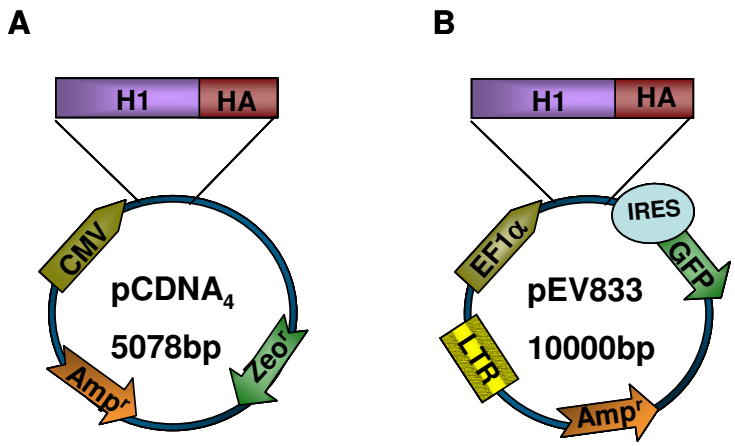


Figure. 8. Schematic representation of the vectors used for exogenous expression of H1 variants.

(A) pCDNA4 vector: the H1-HA variant cassette is under the control of the CMV promoter. Selection is made by the zeocine resistant gene.

(B) pEV833 vector: a lentiviral derived vector containing the LTR from HIV virus. The viral promoter EF1alpha controls the expression of the H1-HA subtypes. GFP gene is the selection marker in this vector.

1.3.1. Stable T47D-MTVL cell lines expressing H1-HA variants.

T47D-MTVL cell line was infected with each of the lentiviral pEV833 vectors containing one somatic H1 variant fused to an HA tagged and a GFP marker gene. Cells were sorted for GFP-positive fluorescence after infection. Populations were analyzed for the expression levels of the exogenous proteins (Figure 9). Western blot analysis showed that the H1-HAs subtypes were expressed and the levels were lower than the endogenous protein. We decided not to increase the levels of expression in order to avoid possible toxic effects because of the overloading of linker histones. As far as we have explored, cells were indistinguishable in morphology and proliferation behaviour from wild-type cell line.

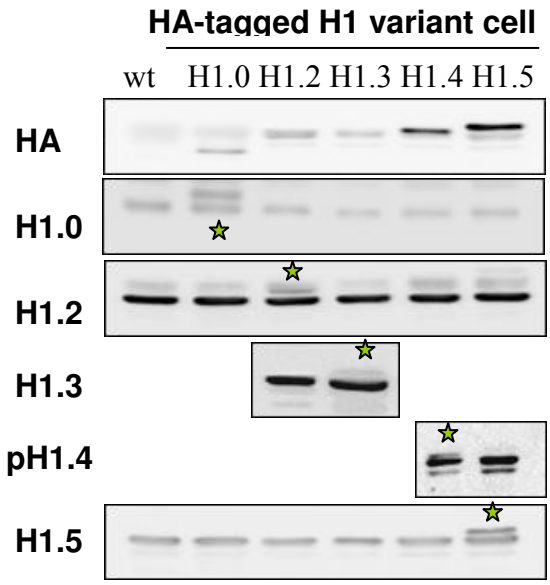


Figure 9. Analysis of the expression of H1-HA variants in T47D-MTVL cell line.

H1 preparations from each H1-HA expressing cell lines were analyzed by western-blot against HA and all the collection of H1 variant specific antibodies.

2. Study of the different role of H1 variants in a breast cancer cell line.

2.1- Characterization of the inducible H1 depleted cell lines.

2.1.1- Inducible and specific shRNA mediated inhibition of H1 subtypes.

In order to explore the specificity of the different H1 subtypes on gene expression control and cell proliferation in breast cancer cells, we generated T47D-derived stable cell lines lacking expression of each of the H1 variants specifically. For that purpose, we used a reported inducible shRNA expression system described previously.

Depletion of the different H1 variants was analyzed by Western blot with specific antibodies. Western blot analysis of H1-extracted sample from each knocked-down cell line, after six days of treatment with Dox, revealed a consistent and specific reduction of every H1 variant (Figure 10A). No significant compensation effects by other H1 subtypes were observed in any case at protein level, except a slight H1.0 increase after H1.4 depletion.

The inhibitory effect was also measured by reverse transcription and real-time PCR (RT-qPCR) analysis of H1 variants gene expression before and after Dox treatment for all the cell lines generated (Figure 10B). This showed specific reduction of the targeted gene between 60% and 90% depending on the variant, and some increase in H1.0 mRNA in both H1.2 and H1.4 knocked-down cell lines.

All these results were confirmed by electrospray mass spectrometry (Figure 11). Acid extraction of H1 sample from each cell line treated with Dox, were sent to the proteomic facility. Although this technique is not quantitative it permits to compare among samples the presence of the different variants in a qualitative way by its differential molecular weight. The first panel of figure 9 shows a chromatogram from the acid extracted H1 of the non-treated H1.2 knocked-down cell line. H1.0 is the first variant to elute (20730 Da), however its relative abundance in comparison to the rest is so low that appears most of the times hidden by the background of the technique. H1.2 elutes second, the major peak corresponds to the mass of the unmodified histone (21270 Da) and it is followed by several smaller peaks that correspond to mostly phosphorylated forms of the histone. The same happens with the other variants, appearing in the following order: H1.4 (21775 Da), H1.3 (22260 Da) and H1.5 (22489), and they are always represented by a major peak, the unmodified protein, followed by the phosphorylated forms. In the subsequent panels of figure 9 it is shown the decrease in the peaks of the knocked-down H1 isoform in that particular cell line when treated for six days with Dox. The chromatogram of the inhibition of H1.2 shows also lower levels of H1.3 and H1.4, but probably we do not have to pay attention as the technique is not quantitative. In

fact, western analysis does not show such depletions of other isoforms (Figure 10A).In the case of H1.5 the decrease is not very clear although it has to take into account the higher relative abundance of that particular isoform in respect to the others. Probably the technique is not enough quantitative to see it.

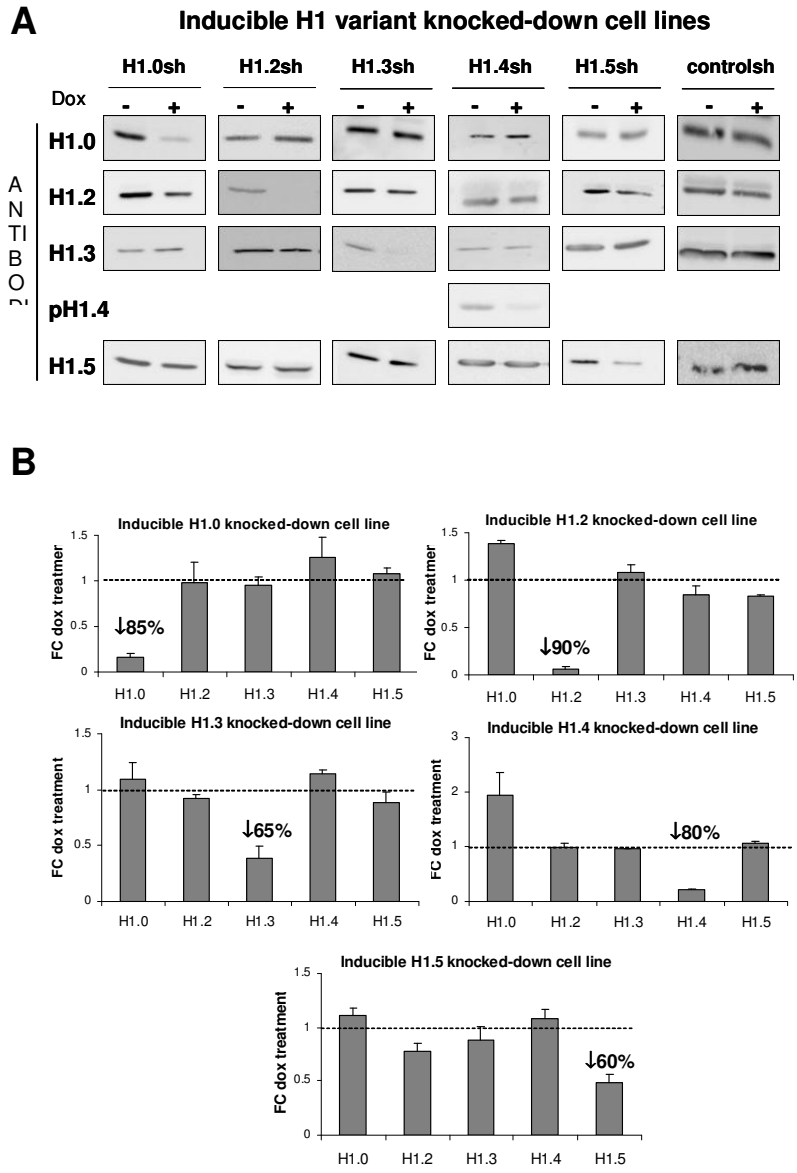


Figure 10. Inducible RNA interference-mediated knock-down of histone H1 variants.

T47D derivative cells stably infected with the lentiviral inducible system for the expression of shRNAs against each of the human histone H1 variants indicated were treated for 6 days with Dox or left untreated.

(A) Western blot with isoform-specific antibodies on histone H1 preparations obtained by perchloric acid extraction from each knocked-down cell line.

(B) Inhibition of H1 expression was tested by RT followed by real-time PCR with oligonucleotides specific for the different human H1 variant genes. Data is expressed as fold change of H1 gene expression corrected by GAPDH in response to Dox compared to untreated.

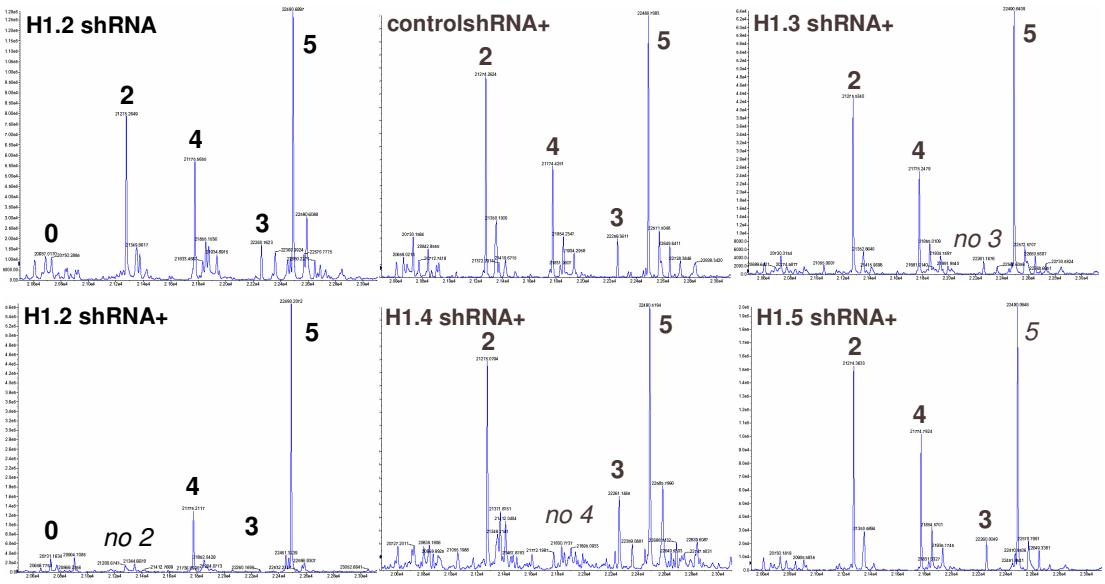


Figure 11. Inhibition of H1 variants followed by electrospray mass-spectrometry (ES-MS).

Extracted H1 histones from knock-down cells treated (+) or not with Dox for 6 days were analyzed by ES-MS.

Once we generated the inducible knocked-down cell lines and checked the inhibition we decided to characterize them in detail in terms of cell proliferation, chromatin structure and gene expression.

2.1.2- Cell proliferation effects after H1 subtype depletion.

Depletion of the different H1 subtypes upon six days of Dox treatment caused differences in growth rate among H1 variant knock-downs, in particular, H1.2 and H1.4 depleted cells failed to reach confluency. A reduction in the number of cells attached to the culture flask and changes in the morphology of the remaining cells, specially for H1.4, were observed (Figure 12A). To quantify possible differences in growth rates among the cell lines, we mixed 1:1 each of the shRNA expressing cell lines (RedFP and GFP-positive upon Dox treatment) with parental T47D cells (RedFP and GFP-negative), and we followed the proportion between the two populations along time, in culture in the presence of Dox, by FACS (Figure 12B). If affected by H1 depletion, the percentage of fluorescent cells (H1 knock-down) would decrease and parental cells (non-fluorescent) would be enriched in the culture. Slow progression of H1.4 knocked-down cells was seen at six days after Dox addition and of H1.2 depleted cells at day 9 (Figure 12C). At day 12, H1.5 depleted cells were also less abundant than parental. In addition, at day 6, H1.4 cells had changed morphology towards a necrotic phenotype (Figure 12A).

To understand the causes of these proliferation defects, the cell cycle profile of the different cell lines was examined by FACS analysis of propidium iodide-stained cells six days after Dox addition and compared to untreated cells. The knocked-down cells lines for H1.0 and H1.3 did not show any significant difference in the cell cycle profile in comparison to uninduced cells or to the controlsh cell line treated with Dox. On the contrary, both H1.2 and H1.4 depleted cells exhibited a dramatic reduction in the S phase cell population while H1.5 depletion caused a slight decrease of S phase (Figure 12A). Interestingly, in the H1.2 knock-down, an increase in the G1 peak over controls and a concomitant decrease in G2 peak were observed. That is, depletion of H1.2 induced G1 arrest. However, in the H1.4 knock-down, a small increase of G2 and almost no change in G1 was observed. Although the cell cycle pattern was not very dramatic, the increase in subG1 peak indicates a high level of mortality in the H1.4 depleted cells (Figure 12B), in agreement with cell growth experiments.

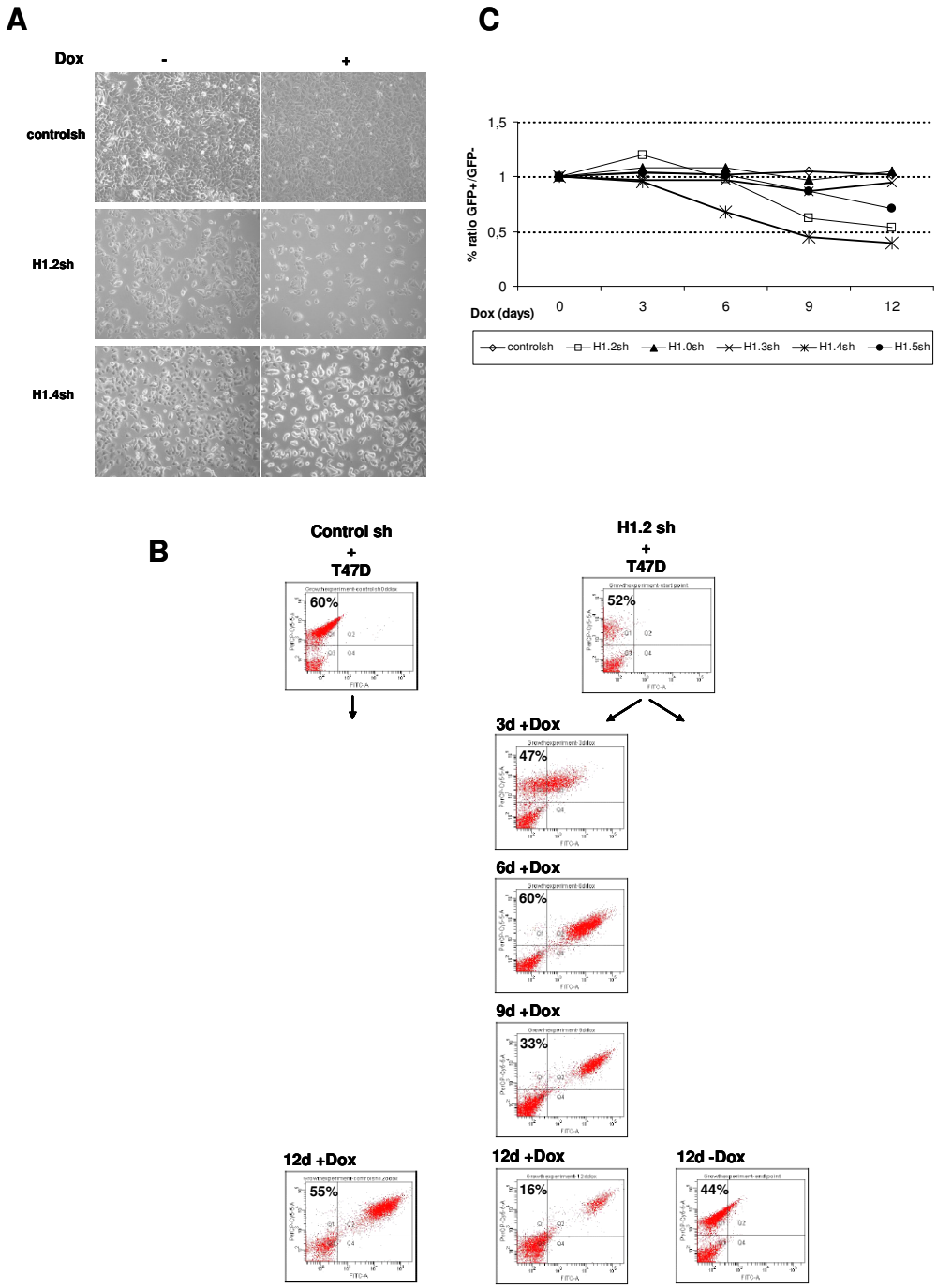


Figure 12. Inhibition of H1 variants causes different effects on cell proliferation.

(A) Optical microscopy of H1 knocked-down cell cultures. T47D cells infected with the inducible shRNA-expression system against H1.2, H1.4 or empty plasmid were treated for 6 days with Dox or left untreated and observed at the optical microscopy.

(B) Experimental procedure followed to measure the effect of H1 depletion on cell proliferation. Each H1 variant knock-down cell line (RFP and GFP-positive) was mixed 1:1 with parental T47D cells (RFP and GFP-negative) and treated with Dox. Every three days, cells were split and the percentage of RFP/GFP-positive cells measured by FACS. The figure summarizes the case for H1.2 inhibition. The

percentage of remaining RFP-positive cells is indicated. Same cell mixture left untreated in parallel, or control shRNA cells were submitted to the same procedure as controls.

(C) Graphical representation of the data obtained in the experiment described previously for all the H1 variant knocked-down cell line. Data is expressed as percentage of variation of the proportion RFP/GFP-positive versus RFP/GFP-negative cells along time respect to the initial seeding proportion.

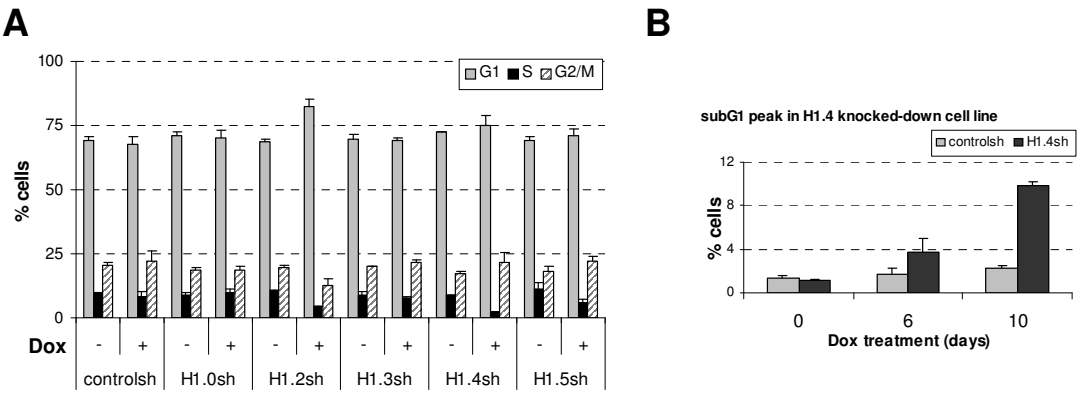


Figure 13. Inhibition of H1 variants causes different effects on cell cycle progression.

(A) Cell cycle profile after propidium iodide (PI) staining of H1 variant knock-down cell lines grown for six days in the presence or absence of Dox. Data is expressed as percentage of cells in G1, S and G2/M cell cycle phases.

(B) H1.4 knocked-down and control cells grown in the presence of Dox for 6 and 12 days were analyzed by FACS after PI staining to measure the percentage of cells in subG1phase as indicative of apoptosis.

2.1.2.a- H1.4 is essential for survival of T47D.

Because of H1.4 depletion leads to high mortality (Figure 13B) and changes in morphology of the cells (Figure 13A), we decided to perform apoptosis analysis in order to understand what it is occurring in this cell line. Experiments performed to measure apoptotic cells (such as formation of apoptotic DNA ladder, anexin V binding, or TUNEL assays), have failed to find differences between H1.4 and control shRNA cells. Similarly, no markers of cell senescence have been identified (Figure 14). Based on these results, we conclude that H1.4 knocked-down cells show a cell death phenotype, probably by necrosis, although more accurate analysis has to be done.

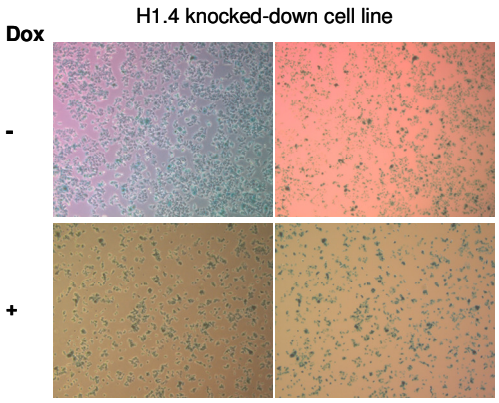


Figure 14. Beta-galactosidase staining as an indicator of senescence status of H1.4 KD cell line. No differences were observed in the Dox treated H1.4 knocked-down cells. In the right image cells were visible while in the left images only the staining was recorded.

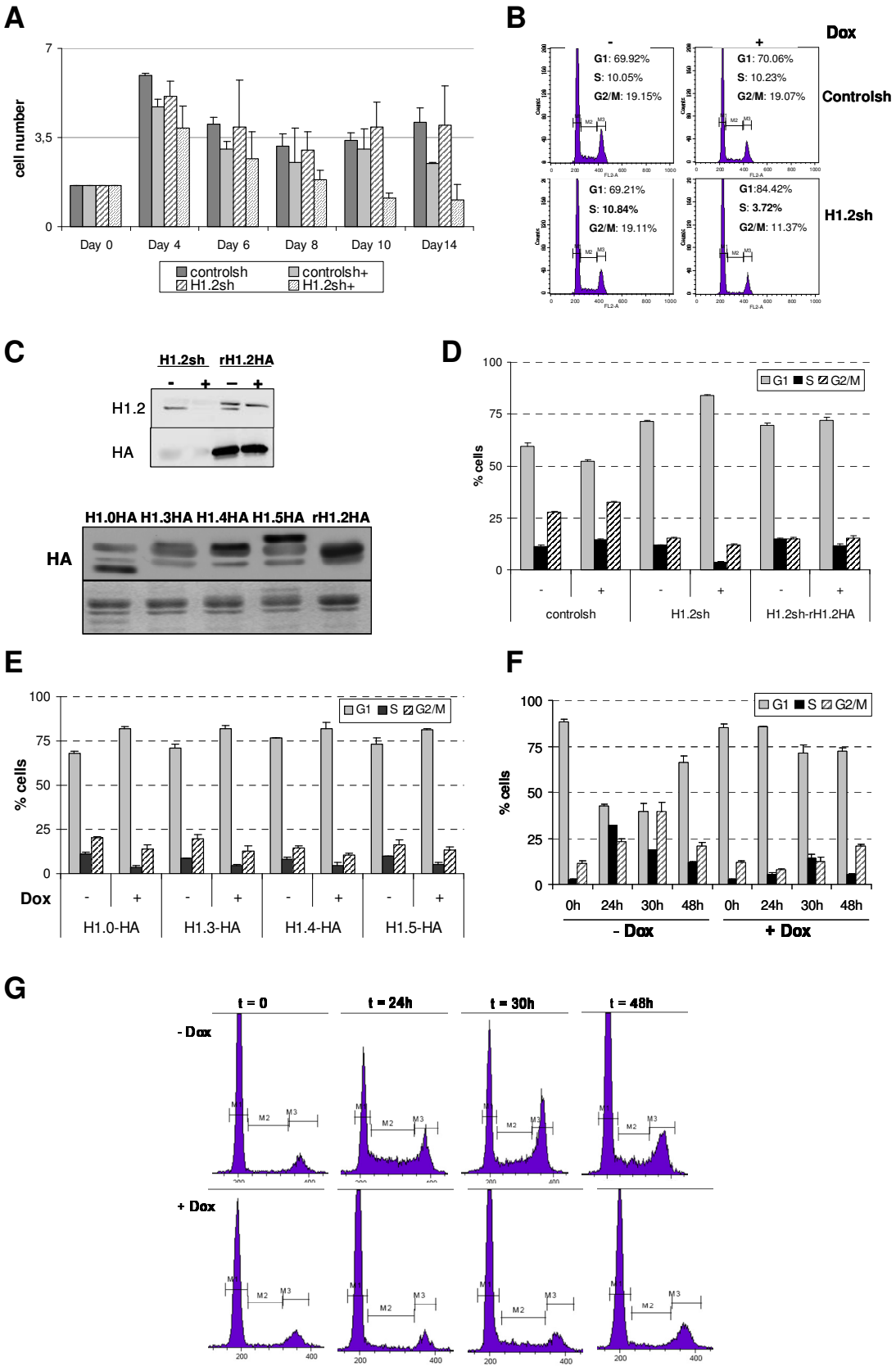
2.1.2.b- H1.2 depletion causes cell cycle arrest in G1.

As we have mentioned previously it seems that depletion of H1.2 causes a G1 arrest in a breast cancer cell line such as T47D. Because of this result, we decided to explore with more detail the cell cycle phenotype observed. Growth rate for depletion of H1.2 was monitored specifically by counting the number of cells in the population every 3 days. From day 6 of Dox treatment there is a progressive decrease of the H1.2 depleted population that is not able to follow the growth rate of other populations such as the non- treated or the controlsh treated also with Dox (Figure 15A). It is at that point, six days of Dox treatment, when propidium iodide staining experiments showed a clear arrest in G1 phase (Figure 15B).

In order to demonstrate that the cell cycle arrest was specific for the depletion of the H1.2 variant, we stably integrated into the H1.2 knocked-down cell line a lentiviral vector for the expression of a shRNA-resistant H1.2 encoding gene C-terminally fused to the HA peptide. The levels of H1.2-HA protein were similar to the endogenous histone variant as shown by Western

blot analysis (Figure 15C). Cell cycle analysis demonstrated that the G1 arrest observed in the H1.2 depleted cells was reverted when the H1.2-HA protein was present (Figure 15D). Moreover, to exclude that the phenotype was not due to a reduction in the total H1 content we performed the experiment expressing ectopically each of the other HA-tagged H1 variants in the inducible H1.2 knock-down cell line (Figure 15C). As Figure 12E exhibits, any of the other variants was able to revert the G1 arrest. All together indicated that H1.2 variant is specifically required for normal cell cycle progression and its depletion causes cells to accumulate in G1 phase.

In addition, we studied the progression of cell cycle after a serum-starvation block in G1. H1.2 knock-down (KD) cells treated or not with Dox for 6 days were serum-starved for the last two days. After serum addition, the cell cycle profile was determined at 24, 30 and 48 h (Figure 15F and G). Dox untreated cells progressed along the cycle, with a maximum of cells in S phase at 24 h, in G2/M at 30 h, and in G1 again at 48 h. Dox-treated cells remained arrested in G1-phase, with little increase of cells in S-phase at 30 h after serum addition. This indicated that the G1 block caused by H1.2 depletion is strong and very few cells are able to escape and progress through the cell cycle.


A
cell number
7
3,5
0
Day 0
Day 4
Day 6
Day 8
Day 10
Day14
controlsh
H1.2sh
controlsh+
H1.2sh+
B
Dox
-
+
Controlsh
H1.2sh
G1: 69.92%
S: 10.05%
G2/M: 19.15%
G1: 70.06%
S: 10.23%
G2/M: 19.07%
G1: 69.21%
S: 10.84%
G2/M: 19.11%
G1:84.42%
S: 3.72%
G2/M: 11.37%
C
H1.2sh
rH1.2HA
H1.2
HA
H1.0HA H1.3HA H1.4HA H1.5HA rH1.2HA
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G1 S G2/M
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H1.3-HA
H1.4-HA
H1.5-HA
F
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G1 S G2/M
0h
24h
30h
48h
- Dox
+ Dox
G
t = 0
t = 24h
t = 30h
t = 48h
- Dox
+ Dox

Figure 15. Specific depletion of H1.2 causes G1 arrest.

(A) Representation of the growth rate of controlsh and H1.2 KD cells along Dox treatment. Every 2 days cells were trypsinize and counted. The same number of cells was seeded again.

(B) Cell cycle profile after propidium iodide (PI) staining of H1.2 variant KD and control cells grown in serum-containing media for six days in the presence or absence of Dox. The percentage of cells in G1, S and G2/M cell cycle phases is indicated.

(C) Expression of HA-tagged H1 variants in H1.2 knocked-down cells. shRNA-resistant H1.2 and the rest of H1 variants, fused to HA peptide at their C-termini, were introduced by lentiviral infection into shH1.2 cells. Expression was measured with an anti-HA or H1 variant-specific antibodies by Western blot. Cells were treated with Dox for 6 days when indicated. Ponceau staining of transferred H1 material is shown as a loading standard.

(D) G1 arrest in H1.2 knocked-down cells is reverted by stable expression of recombinant HA-tagged, shRNA-resistant H1.2,

(E) but not by expression of other H1 variants.

(F) Cell cycle profile after propidium iodide (PI) staining of H1.2 KD cells cultured for six days in the presence or absence of Dox. Cells were kept in serum-free media for the last two days to arrest cells in G1 and then released by serum addition. The percentage of cells in G1, S and G2/M cell cycle phases is indicated.

(G) Graphical representation of the previous (F) experiment.

2**.1.2.c-** DNA damage response of H1.2 and H1.4 knocked-down cell lines.

DNA damage response is tightly related to cell cycle progression, because activation of proteins involved in cell-cycle arrest also leads to induction of genes that participate in DNA repair and apoptosis. DNA damages can perturb the cellular steady-state equilibrium and activate or amplify certain biochemical pathways that regulate cell growth and division and pathways that help to coordinate DNA replication with damage removal. Because we obseved a cell cycle arrest phenotype in one of our knocked down cell lines, we decided to examine if the DNA damage response was also affected by the lack of H1.2.

Hydroxyurea (HU), an RNA inhibitor, and methyl methanosulfonate (MMS), a damaging agent, block cycling cells at different stages. HU blocks replication and stops cells in S-phase whereas MMS produces G2/M arrest. We investigated how these drugs affected the cell cycle profile of ±Dox-treated H1.2 and H1.4 KD cells. While the cell cycle profile of H1.4 KD in response to HU or MMS was practically indistinguishable in the presence or absence of Dox, H1.2 KD cells treated with Dox remained accumulated in G1 whether HU or MMS was present or not (Figure 16). Only a small increase of cells in S-phase in the presence of HU or MMS

occurred, indicating that residual cycling occurs. These results indicated that H1.2 depletion causes a strong G1 arrest phenotype and H1.4 KD cell cycle is not greatly affected in the few surviving cells.

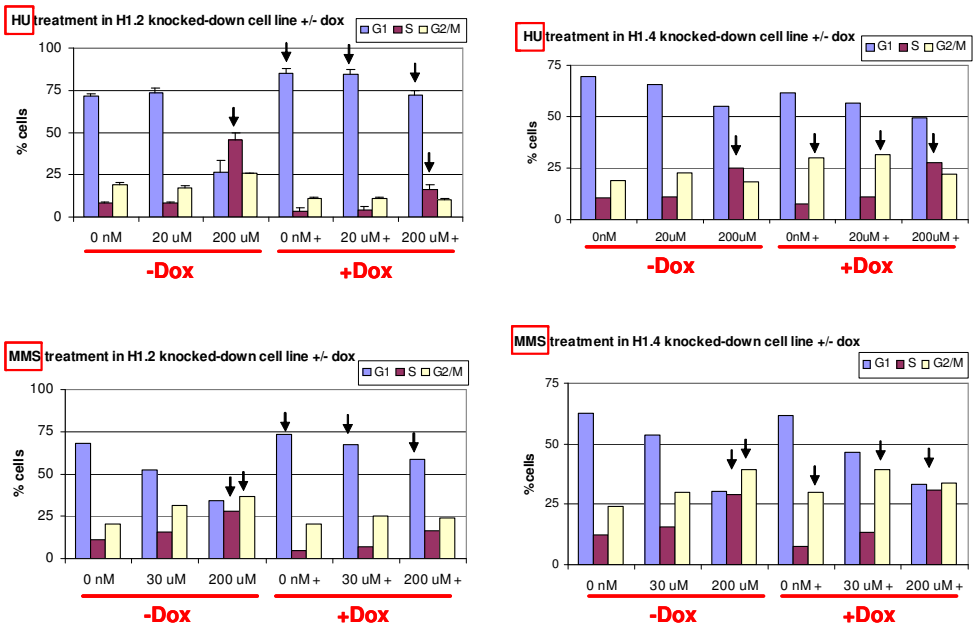


Figure 16. Cell cycle profile of H1 knock-down cells in response to treatment with different inhibitors. H1.2 and H1.4 KD cell lines were treated or not with Dox for 6 days. Hydroxyurea (HU) or methyl methanosulfonate (MMS) were added at the indicated concentrations 12 h before analysis of the cell cycle profile by FACS measurement of PI-stained cells.

2.1.4.d- Variant-specific effects of H1 depletion in cell progression are not restricted to T47D cells.

In order to investigate whether the effect of H1.2 and H1.4 deletion on cell proliferation was specific to the breast cancer cell line T47D used or is a more extended phenotype, we introduced the inducible shRNA expression system in a different breast adenocarcinoma cell line (MCF7), a non-tumoral breast epithelial cell line (MCF10A), a cervical adenocarcinoma cell line (HeLa) and an embryonic kidney cell line (293T). Efficient H1.2 and H1.4 depletion in response to Dox treatment was obtained for all cell lines (Figure 17A and B).

Taking advantage of the system established for T47D to measure differences in growth rates, we performed the same experiment in MCF7, MCF10A and 293T cell lines. We mixed 1:1 each of the shRNA expressing cell lines (RedFP and GFP-positive upon Dox treatment) with

each parental cells (RedFP and GFP-negative), and we followed the proportion between both populations treated with Dox along time, by FACS (Figure 17C). As we have explained before, the percentage of GFP+ cells (H1 KD) would decrease and parental cells (GFP-) would be enriched in the culture if cell growth is affected by H1 depletion. H1.2 depletion caused a clear defect on MCF7 cell growth, whereas H1.4 inhibition affected to MCF10A cell line. Microscopy images taken after six days of Dox treatment confirmed these results except for the MCF10A-H1.2sh cell line that showed a clear decrease in the number of cells (Figure 17D). This observation could be explained by a possible toxic effect of Dox in MCF10A cells. During the growth rate experiment we observed that the total viability of the MCF10A populations was decreasing along Dox treatment. H1.4 depletion was obtained for HeLa and MCF7 (Figure 17B), but no proliferation defects were observed.

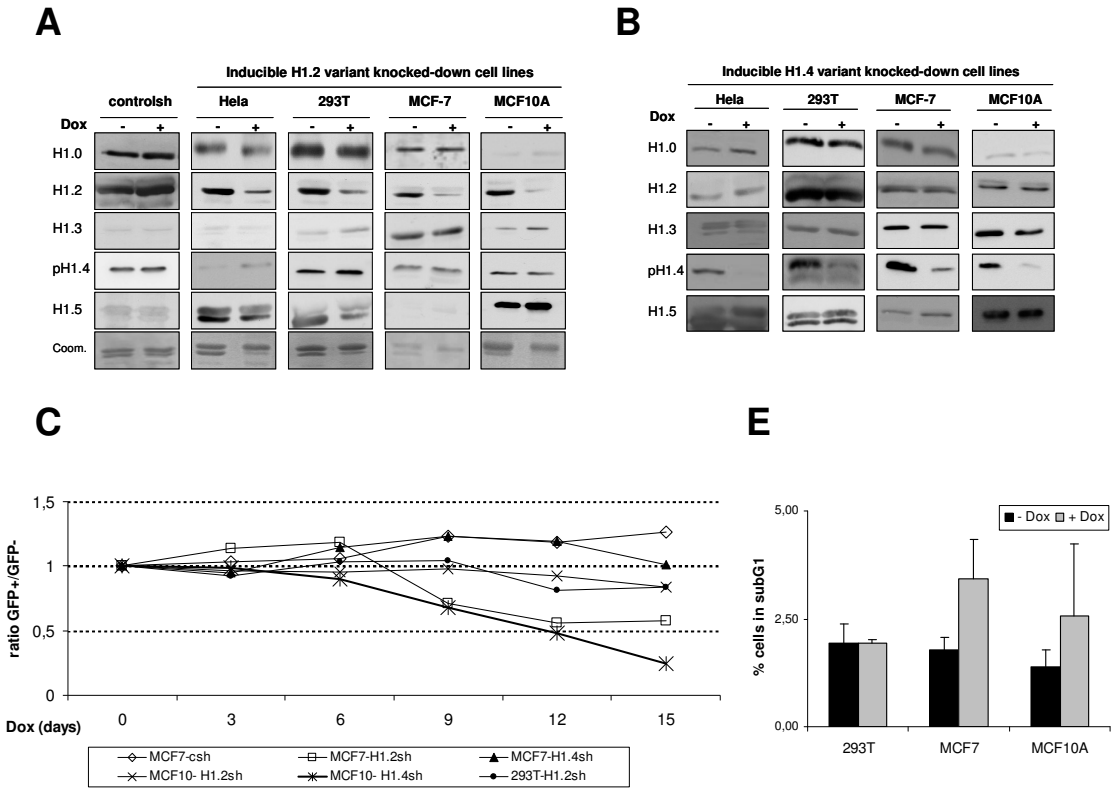
Analysis of the cell cycle profile in H1.2 knocked-down cell lines failed to show G1 arrest in any of the cell lines analyzed, but a clear increase in the subG1 apoptotic peak was observed in MCF7 cells (Figure 17E). In the case of MCF10A cells, the error bar invalidates the result. That is, proliferation of MCF10A, HeLa and 293T cell lines was not affected by H1.2 depletion. However, H1.2 depletion has consequences on proliferation not only of T47D, but also on other tumoral breast epithelial cell lines such as MCF7. In addition to this, H1.4 depletion causes also a defect in cell progression in both T47D and MCF10A cell lines.

Figure 17. **Variant-specific effects of H1 depletion in cell progression are not restricted to T47D cells.** HeLa, 293T, MCF7 and MCF10A cells were infected with the inducible shRNA-expression system to inhibit expression of **(A)** H1.2 or **(B)** H1.4. Linker histone depletion was tested by western blot with specific antibodies.

(C) Graphical representation of the data obtained in the cell proliferation experiment described in the text for all the H1 variant knocked-down cell line. Data is expressed as ratio of variation of the proportion RFP/GFP-positive versus RFP/GFP-negative cells along time respect to the initial seeding proportion.

(D) Cell lines indicated infected with the inducible shRNA-expression system against H1.2 were treated for 6 days with Dox or left untreated and observed at the optical microscopy.

(E) H1.2 knocked-down cells grown in the presence of Dox for 6 days were analyzed by FACS after PI staining to measure the percentage of cells in subG1-phase as indicative of apoptosis.


A
Inducible H1.2 variant knocked-down cell lines
controlsh
Hela
293T
MCF-7
MCF10A
Dox
H1.0
H1.2
H1.3
pH1.4
H1.5
Coom.
B
Inducible H1.4 variant knocked-down cell lines
C
ratio GFP+/GFP-
Dox (days)
MCF7-csh
MCF7-H1.2sh
MCF7-H1.4sh
MCF10- H1.2sh
MCF10- H1.4sh
293T-H1.2sh
E
% cells in subG1
- Dox
+ Dox
293T
MCF7
MCF10A
D
Inducible H1.2 KD cell lines
Inducible H1.4 KD cell lines

2.1.3- Global changes in chromatin structure by H1 subtype depletion.

2.1.3.a- Nucleosome spacing alteration is observed in the H1.2 knocked-down cell line.

Linker histone H1 is a key component in the organization of the chromatin in eukaryotic cells. Recently, it has been published that a reduction of 50% of H1 content produces a reduction in the nucleosome spacing of nuclear bulk chromatin from the triple-H1 null mouse embryonic stem (ES) cells (Fan et al. 2005). Consequently, we have investigated alterations in chromatin structure from our different H1 variant T47D knocked-down cell lines treated or not with Dox. For that purpose, we analyzed the nuclesome spacing by *micrococcal* nuclease (MNase) digestion of isolated nuclei from each cell line (Figure 18A). H1.2 depletion, but not depletion of other H1 variants, caused a striking reduction in the spacing between nucleosomes. The calculated nucleosome repeat length (NRL) was decreased from 189 to 181 upon H1.2 depletion (Figure 18C). Surprisingly, H1.2 only represents approximately 20% of the total H1 content in T47D cells. According to the level of inhibition achieved, we estimate that H1 content was reduced approximately 18%. Moreover, we showed that this alteration is specific for the lack of H1.2 because stable expression of HA-tagged, shRNA-resistant H1.2 (rH1.2-HA) in the H1.2 KD cell line reverted the reduction on nucleosome spacing caused by endogenous H1.2 depletion (Figure 18B).

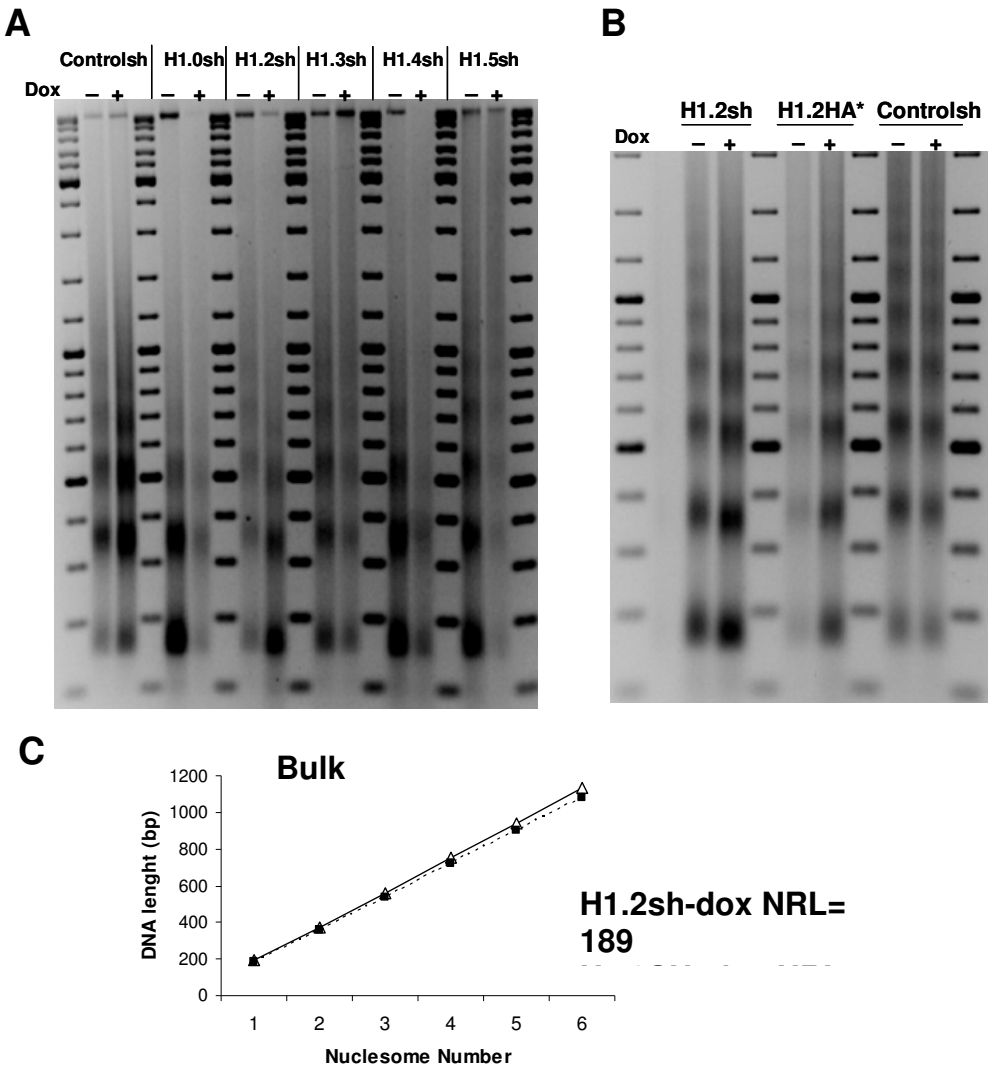


Figure 18. H1.2 knock-down causes a reduction in nucleosome spacing.

(A) Nuclei from H1 variant knock-down cells treated or not with Dox for 6 days were treated with MNase and the profile of bulk chromatin was analyzed in gel electrophoresis to calculate the nucleosome repeat length (NRL).

(B) Complementation of reduced nucleosome spacing in H1.2 knocked-down cells by stable expression of shRNA-resistant H1.2 (rH1.2-HA).

(C) Plot of nucleosome number versus DNA length for MNase-digested bulk chromatin of H1.2 knocked-down cells ±Dox shown in (B). This plot has been used to calculate the corresponding NRL. Open triangles and solid lines denote untreated, and solid square symbols and dashed lines correspond to cells treated for 6 days with Dox.

2.1.3.b- H1.2 knocked-down cell line does not cause changes in global histone modifications.

We have also explored global changes in histone modifications. It is possible that the relative quantities of core histone modifications were altered in order to compensate the H1 loss. Certain modifications such as trimethylation of K27 of H3 or acetylation of K12 in H4 can have a role neutralizing the negative charges of DNA in the nucleus and thus maintaining a more compact chromatin status than in the case of H1 depleted chromatin. For this reason we examined several core histone modifications in the H1.2 KD cell line. Interestingly, trimethylation of K27 of H3 or acetylation of K12 in H4 remains unchanged in our H1.2 depleted cells (Figure 19).

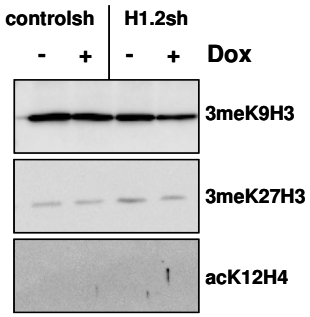


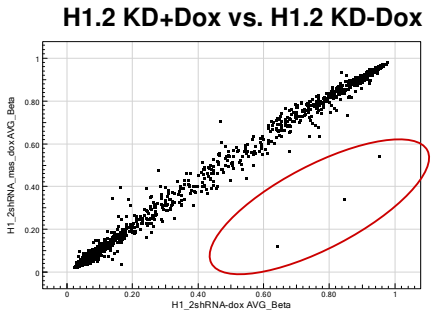
Figure 19. **H1.2 knock-down leaves unchanged several post-translational modifications in core histones** analyzed globally by western-blot with specific antibodies, such as H3 K9 or K27 trimethylation or H4 K12 acetylation.

2.1.3.c- Depletion of H1.2 does not alter DNA methylation pattern.

Previous studies in the triple knock-out cells for H1 showed some changes in DNA methylation pattern of some particular imprinted genes. To investigate this possibility in our H1.2 depleted model we compared controlsh and H1.2 depleted cells for the genomic DNA methylation profile by using a methylation array from Illumina containing 800 genes related to cancer in collaboration to Dr. Esteller's laboratory. As it was previously suggested no global changes in DNA methylation were observed. However, depletion of H1.2 in our cell model does not lead to specific changes in DNA methylation of particular genes. In this type of experiments it is considered that a gene changes its level of methylation when goes from 0-40% (hypomehylated) to more than 50% (hypermethylated) or in the other way around. According to these criteria the scatter plot for the samples showed only three genes hypomethylated when H1.2 was depleted by Dox addition(Figure 20A). If we look in detail the changes are mainly due to the high dispersion of the H1.2 KD cell line non-treated with Dox in comparison to the controlsh ± Dox (Figure 20B). In conclusion, we were not able to see

changes in DNA methylation profiles when H1.2 is depleted. Further studies should be considered to discard completely this possibility.

A



Gene Symbol	controlsh - Dox	controlsh + Dox	H1.2sh KD - Dox	H1.2 KD + Dox
FMR1	0,590	0,279	0,952	0,538
NEFL	0,282	0,190	0,844	0,342
RUNX1T1	0,153	0,119	0,644	0,121

B

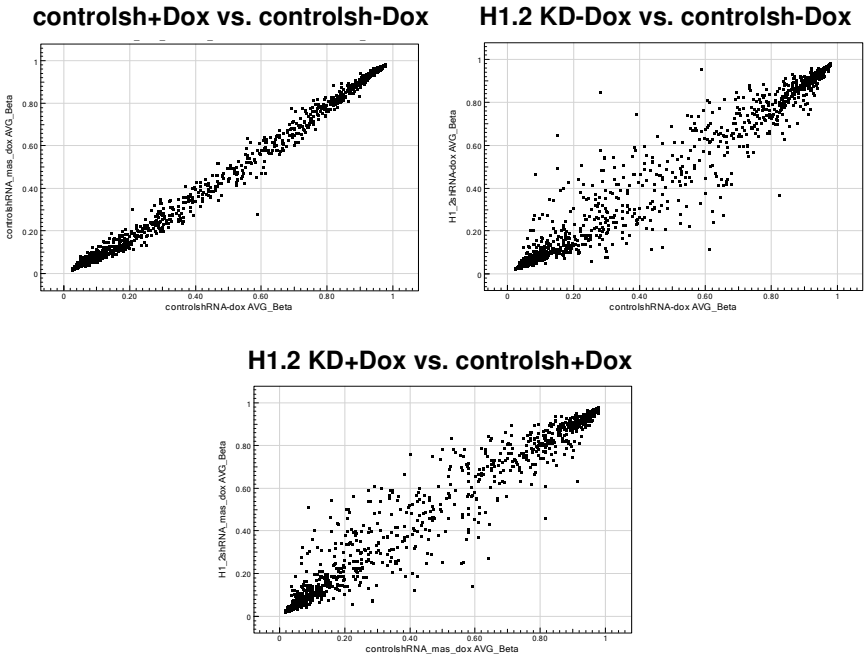


Figure 20. Analysis of DNA methylation pattern in H1.2 KD ± Dox and controlsh ± Dox. Genomic DNA was extracted for both cell lines treated or not with Dox for 6 days and hybridized to a methylation array containing 800 genes related to cancer from Illumina.

(A) Scatter plot for H1.2 knocked-down cells treated with Dox versus H1.2 non-treated. Only three genes appear to change significantly (red circle). In the below panel is shown the data for these three genes. The high levels of the H1.2 KD –Dox cell line in comparison to the other cell lines are pointed in blue.

(B) Scatter plot for other comparisons indicated. It is remarkable the big dispersion measured in the H1.2 KD –Dox in comparison for instance with controlsh –Dox.

2.1.4- Effects on gene expression in the H1 knocked-down cell lines.

Histone H1 has been considered a transcriptional repressor, suggesting that the presence of H1 may function as an obstacle for the transcription machinery. Nonetheless, an active role (positive or negative) of H1 on gene expression control has also been proposed. To further investigate this, we have studied the effect of H1 variants depletion on global gene expression.

For that purpose, we performed microarray experiments using a customized microarray containing PCR products of about 800 cDNA clones involved in breast cancer, cell cycle, transcription factors and chromatin related proteins (See Material & Methods). Depletion of H1 variants caused the alteration on the expression of a limited number of genes, different in each knocked-down H1 variant cell line.

Reduced H1.2 content leads to highly specific changes in gene expression.

Gene expression profiles from H1.2 knocked-down and controlsh cell lines treated or not with Dox, were compared in culture conditions of serum deprivation for 24 hours to synchronize cells in the same cell cycle phase to avoid comparing cells arrested in the cell cycle (H1.2 KD +Dox) with cells normally cycling (H1.2 KD –Dox). After processing the data according to our criteria for spot intensity relative to background and reproducibility (see Materials and Methods), we found that 70 genes differed more than 1.4 fold ($q \leq 0.1$), including the H1.2 gene, as expected, that is down-regulated -7.67 fold (Table 2). The majority of the genes were down-regulated and only 11 were activated in comparison to the control cell line. Interestingly, cell cycle related genes were over-represented and most of them repressed such as ccnb2, cdc2, cdc20, cdkn3 and mad2L1. Approximately 30% of the altered genes were cell cycle regulators, taking into account that this functional category only represents the 17% of the genes included in the customized array. Thus, data suggests that the expression profile of the H1.2 depleted cell line is related to the cell cycle phenotype identified previously.

Gene symbol	Average fold-change and significance [a]				GO [b]
	Control sh ±Dox		H1.2 sh ±Dox		
Genes down-regulated in the presence of Dox					
HIST1H1C	-1.23	ns	**-7.67**	**	
ITGB1	1.19	ns	**-2.50**	**	
HSP90AA1	-1.23	ns	**-2.42**	**	
CDKN3	1.04	ns	**-2.38**	**	CC
CDC2	-1.16	ns	**-2.31**	**	CC
NUSAP1	1.02	ns	**-2.13**	**	CC
TOP2A	1.08	ns	**-2.13**	**	
AURKB	1.02	ns	**-1.95**	**	CC
LPXN	1.12	ns	**-1.91**	**	
BIRC5	-1.12	ns	**-1.88**	**	CC
MELK	-1.22	ns	**-1.79**	**	
KNTC2	1.20	ns	**-1.76**	**	CC
CDC23	-1.03	ns	**-1.74**	**	CC
EXO1	-1.05	ns	**-1.72**	**	
SART3	-1.06	ns	**-1.71**	**	
RPL7	-1.32	ns	**-1.70**	*	
CKS2	-1.02	ns	**-1.67**	**	CC
PRC1	-1.09	ns	**-1.64**	**	CC
KRT5	-1.80	ns	**-1.62**	**	
WISP1	-1.04	ns	**-1.62**	**	
FGFR2	1.00	ns	**-1.61**	**	
DIAPH3L	-1.06	ns	**-1.60**	**	
CDC20	-1.09	ns	**-1.59**	*	CC
CDC45L	-1.00	ns	**-1.57**	**	CC
BLM	1.04	ns	**-1.57**	**	
SCGB1A1	1.02	ns	**-1.56**	**	
HSPB2	-1.02	ns	**-1.55**	**	
MAD2L1	-1.08	ns	**-1.54**	*	CC
CCNB2	-1.12	ns	**-1.54**	*	CC
CYBASC3	-1.01	ns	**-1.54**	**	
DTL	-1.03	ns	**-1.54**	*	
RFC3	-1.06	ns	**-1.53**	**	
RBBP8	-1.09	ns	**-1.52**	**	CC
SCGB1C1	-1.10	ns	**-1.51**	**	
MCM6	1.01	ns	**-1.49**	**	CC
BCL2	1.04	ns	**-1.48**	*	CC
SURB7	1.01	ns	**-1.47**	**	
XRCC5	1.01	ns	**-1.46**	**	
WNT10B	1.01	ns	**-1.45**	*	
CDC6	-1.24	ns	**-1.45**	*	CC
FAS	1.06	ns	**-1.45**	**	
C20orf46	-1.01	ns	**-1.44**	*	
SERPINB2	1.04	ns	**-1.44**	*	
AKT3	-1.04	ns	**-1.43**	*	
SHBG	-1.01	ns	**-1.43**	*	
HSD11B1	1.01	ns	**-1.43**	*	
CCNB1	1.05	ns	**-1.42**	**	CC
CKB	-1.12	ns	**-1.42**	*	
TFF1	-1.15	ns	**-1.42**	*	
KRT17	-1.14	ns	**-1.42**	*	
MAP2K4	1.04	ns	**-1.42**	**	
S100A2	-1.01	ns	**-1.41**	*	
KIT	1.13	ns	**-1.41**	*	
PPARG	1.03	ns	**-1.40**	*	
HSD17B8	-1.08	ns	**-1.40**	*	
HSD17B2	1.03	ns	**-1.40**	*	
ZNF533	1.08	ns	**-1.40**	*	
BRCA2	-1.08	ns	**-1.40**	*	CC
NME2	1.06	ns	**-1.40**	*	CC
Genes up-regulated in the presence of Dox					
EGFP	**67.49**	**	**31.23**	**	
C14orf109	-1.02	ns	**2.08**	*	
IFIT2	1.00	ns	**1.80**	*	
NCOA1	1.18	ns	**1.73**	*	
ERBB2	-1.19	ns	**1.73**	*	
CALR	-1.06	ns	**1.71**	*	
MAP4K3	1.10	ns	**1.69**	*	
NRIP1	1.15	ns	**1.67**	*	
CCNG2	1.06	ns	**1.66**	*	CC
SMARCA2	1.12	ns	**1.61**	*	
PGR	-1.20	ns	**1.57**	*	
SMARCA3	1.17	ns	**1.46**	*	

[a] Genes indicated have increased or decreased expression 1.4-fold in the presence of Dox versus in the absence of Dox (6 days).
**, qŠ0.05; *, 0.05Šq Š0.1; ns, non-significant.
[b] Gene onthology: cell cycle-related genes are identified with CC

Table 2. Genes with altered expression in knocked-down H1.2 cell line.

2.1.4.a- Expression of cell cycle-related genes is altered in H1.2-depleted cells.

Expression of some of the genes identified by the microarray experiment were further analyzed by RT-qPCR in a time-course after Dox addition, in rich medium conditions, to be sure that these genes were affected independently of the culture conditions (Figure 20). Some of the genes were repressed (cdc2, cdkn3, rfc3) or activated (cdkn1a) as early as 2 days after Dox addition. Other genes were not repressed until day 6 (cdc20). In a global way, the pattern of expression at six days of Dox treatment confirms the microarray results, independently of culture conditions.

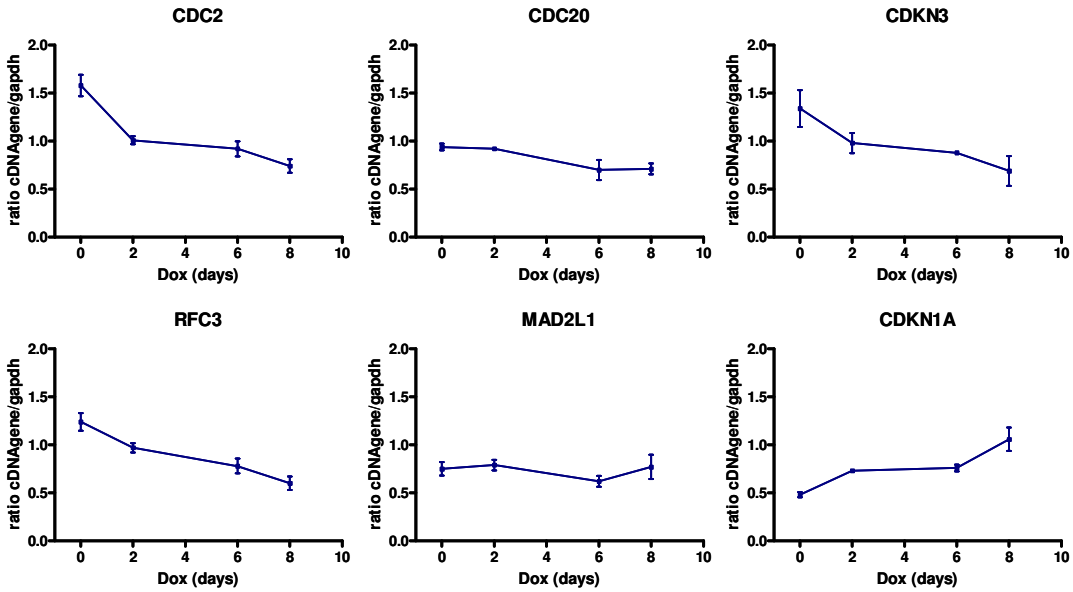


Figure 21. Gene expression profiles along time after Dox addition.

H1.2 KD cells were treated with Dox for the time points indicated and RNA was extracted. Gene expression was measured by RT-quantitative PCR with specific oligonucleotides.

In order to identify a set of genes that could be responsible for the cell cycle phenotype we further explored the relationship between H1.2 depletion and the cell cycle gene expression profile. For that purpose, we have investigated the effect of H1.2 depletion after 12-hour release from a serum-starvation induced G1-arrest block by testing expression of 84 cell cycle-related genes by RT-qPCR using the Super Array (APHS-020) platform. At least 28 genes of this platform were significantly repressed more than 1.5-fold when H1.2 was depleted after 6 days of Dox addition. This repression was not observed in most of the genes in control cells treated with Dox, although there were few of them that showed a minor repression. The repression of the genes was maintained or even higher upon serum induction,

probably because most of these genes were activated after serum addition but cells depleted in H1.2 were not able to respond, then the difference increases significantly. Moreover, only six genes were activated ≥1.5-fold (Table 3) but not at 12 hours. As these genes were already activated without mitogenic signals their activation upon serum addition in control cells minimizes the difference between both conditions. Most of the cell cycle-related genes altered in the microarray data appear also down-regulated in this platform.

	Average fold-change and significance [a]				
	Control sh ±Dox		H1.2 sh ±Dox		H1.2 sh ±Do
Gene symbol	no serum		no serum		12h serum
BIRC5	**-1,73**		**-8,97**	*	**-11,44**
CDKN3	**-2,00**	**	**-6,36**	**	**-9,59**
MKI67	**-2,77**	*	**-6,11**	**	**-10,97**
CDC20	**-1,88**		**-5,66**	**	**-9,23**
GTSE1	**-2,62**	**	**-5,60**	*	**-14,63**
CCNB2	**-1,84**	**	**-4,94**	**	**-7,50**
CDC2	**-1,90**	**	**-3,75**	**	**-7,44**
BRCA1	**-1,77**		**-3,52**	**	**-6,15**
CCNB1	**-1,58**	*	**-3,48**	*	**-5,51**
MCM5	**-1,64**		**-3,29**	**	**-6,09**
MAD2L1	**-1,79**	**	**-3,23**	**	**-6,50**
CKS2	**-1,59**	*	**-3,22**	**	**-5,01**
RBL1	**-1,89**		**-2,76**	**	**-5,74**
PCNA	**-1,79**		**-2,76**	**	**-5,51**
BCL2	-1,07		**-2,61**	**	**-5,10**
MCM3	**-1,50**	*	**-2,51**	*	**-4,43**
RBBP8	1,10		**-2,35**	*	**-3,16**
MCM2	-1,43		**-2,34**	*	**-3,09**
KNTC1	**-1,58**		**-2,28**	**	**-6,26**
CHEK1	-1,48		**-2,23**	**	**-3,83**
RAD1	-1,06		**-2,14**		**-4,49**
CKS1B	-1,40	*	**-2,13**		**-2,69**
CDKN2B	**-2,04**		**-2,11**	*	**-3,72**
MCM4	-1,29		**-1,97**	*	**-4,28**
KPNA2	-1,44	*	**-1,95**	**	**-2,32**
MRE11A	1,02		**-1,93**		**-8,70**
CDK2	-1,35		**-1,90**	**	**-4,76**
CDKN2A	-1,42	*	**-1,82**		**-2,16**
RPA3	-1,46	*	**-1,65**		**-2,31**
TP53	1,15		**-1,57**		1,10
ANAPC4	1,12		**1,54**		-1,30
CDK7	1,26		**1,58**	**	-1,01
CCNT2	1,30		**1,79**		1,04
CDK8	1,07		**1,85**	*	-1,12
CCNG2	1,24		**2,23**	**	-1,39
RBL2	1,18		**2,52**	**	1,05

[a] Genes indicated have increased or decreased expression ≥1.5-fold in the presence of Dox versus in the absence of Dox (6 days).
**, q≤0.05; *, 0.05≤q≤0.1; ns, non-significant.

Table 3. Cell cycle-related genes with altered expression in H1.2 knocked-down cell lines.

Expression of some of the cell cycle-related genes altered in the PCR Array were validated by RT-qPCR (Figure 22A). As there was some repression in the controlsh cells treated with dox we decided to confirm that these genes were specifically down-regulated by the lack of H1.2 expressing the h1.2-HA recombinant protein (sh-RNA resistant). The repression of the genes tested is partially complemented by the exogenous protein as it is shown in Figure 22B. Definitively, cdc2 is the gene that is maintained repressed in H1.2 KD more consistently after serum addition while cdkn1a is constantly activated. In addition, repression of genes such as cdc20, cdkn3, ccnb2 is also very reproducible in the different gene expression analysis performed.

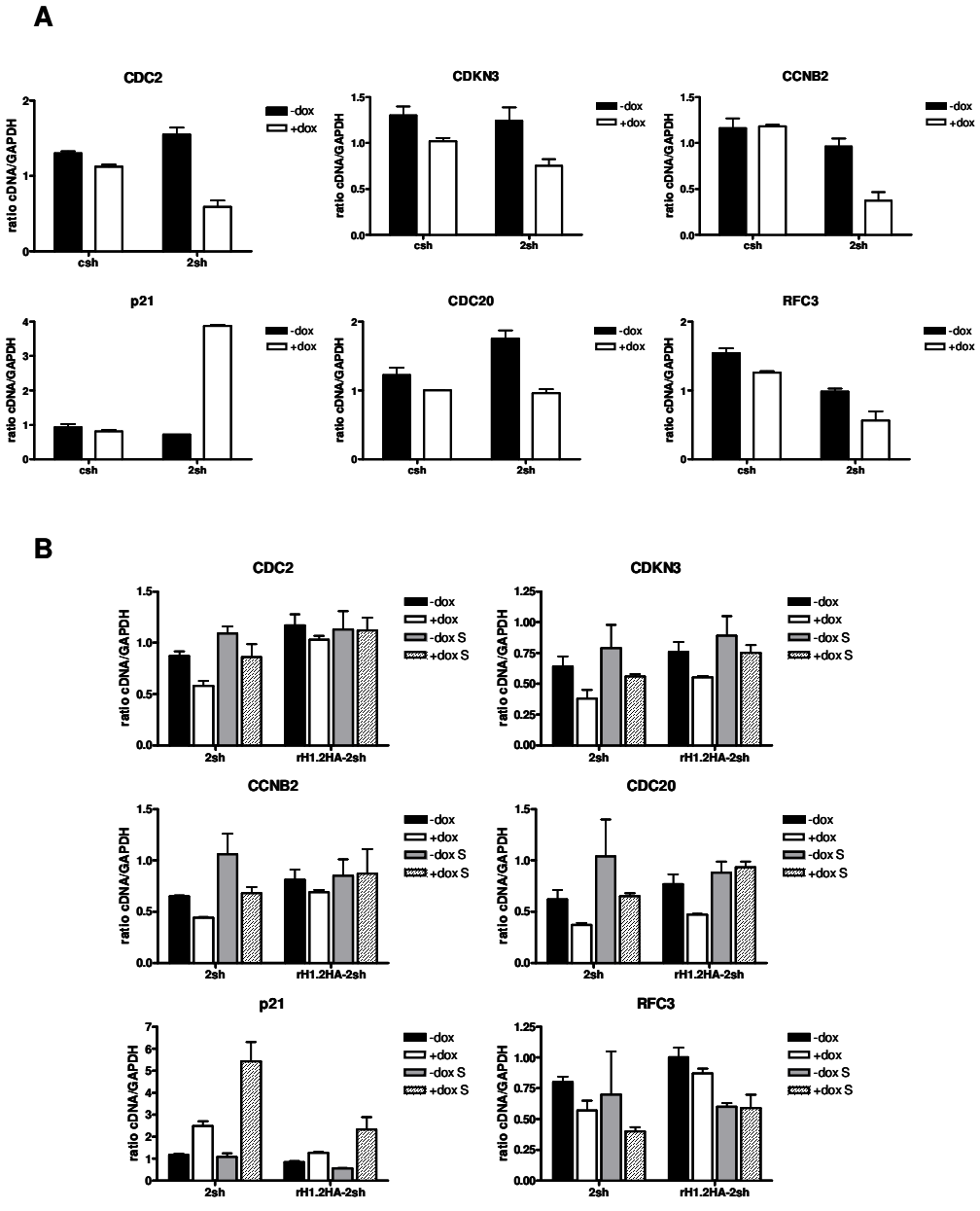


Figure 22. Cell cycle gene expression profiles of H1.2 KD cell line after release from a G1 block. Cells grown for six days in the presence or absence of Dox and without serum for the last two days, were treated with serum and RNA was extracted at time points indicated for gene expression analysis by RT-qPCR.

2.1.4.b-Cell cycle proteins profile in H1.2-depleted cells.

In order to better characterize the G1 arrest caused by H1.2 depletion, we have analyzed accumulation or phosphorylation of several cell cycle regulators in ±Dox-treated H1.2 KD and control cells by western-blot with specific antibodies (Figure 23). Phosphoryation of retinoblastoma protein (Rb) was highly reduced in Dox-treated H1.2 knocked-down cells, but not in control cells. Rb phosphorylation is required for transcription of S-phase genes. In the DNA damage response, p53-dependent induction of expression of the CDK inhibitor $p21^{Cip1}$ is involved in the defect on Rb phosphorylation. In our experiment, accumulation of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ was invariable (Figure 23), although cdkn1a gene expression was increased in H1.2-depleted cells (Figure 20). We cannot rule out that a different player affected ultimately by H1.2 depletion is also involved in Rb phosphorylation. Cdc2 (CDK1) was down-regulated in H1.2-depleted cells and is involved in G1 to S and G2 to M transitions. Surprinsingly the levels of the total protein do not change (Figure 23). To check the phosphorylation level of the protein in order to know if it is active should be considered.

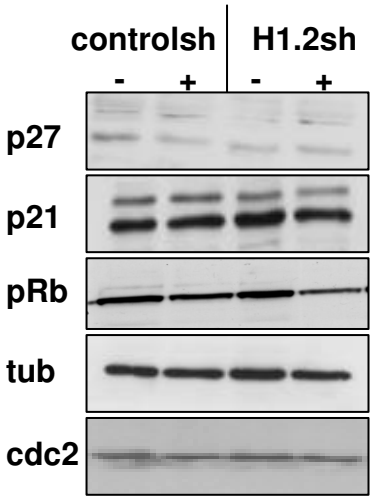


Figure 23. Changes in the accumulation of cell cycle-related proteins upon inhibition of H1.2. Expression of the indicated proteins was measured by western-blot with specific antibodies in cells treated or not with Dox for 6 days.

2.1.4.c- Specific alteration of cell cycle-related genes in other H1 knocked-down cell lines.

We have investigated the pattern of expression of cell cycle-related genes in other knocked-down cell lines such as H1.0 KD and H1.4 KD using the Super Array methodology. Data obtained confirms the existence of specific roles of H1 variants in the regulation of different set of genes. Table 4 summarizes the genes altered in each knocked-down cell line analyzed in comparison to the H1.2 KD and controlsh cell line upon six days of treatment with

doxicycline in serum-deprivation conditions to synchronize cells in the same cell cycle phase, as it was done in previous experiments.

In the H1.0 depleted cell line, 21 genes were up-regulated over 1.5-fold and only 3 down-regulated, although these were also altered in controlsh cells and they would not be considered as real targets. Only a small percentage (8%) of the altered genes coincides with the H1.2 depleted cells.

The analysis of the H1.4 KD cells revealed a high number of genes repressed (31) and just one activated. As H1.4 depleted cells were dying the dramatic cell cycle expression profile could be considered as expected. 44% of the genes were also altered in the H1.2 KD cell line.

Gene symbol	Control sh ±Dox	H1.0 sh ±Dox	H1.2 sh ±Dox [b]	H1.4 sh ±Dox
BRCA1	**-2,12**	**1,93**	**-2,72**	**-2,34**
RAD51	**-1,59**	1,38	**-2,70**	**-1,85**
DDX11	**-1,93**	**-1,97**	**-2,66**	-1,48
CDKN3	-1,15	1,40	**-2,41**	**-2,09**
MCM2	**-1,90**	-1,21	**-2,28**	**-1,78**
BIRC5	**-2,23**	**1,67**	**-2,27**	**-16,27**
CDK2	-1,14	1,28	**-2,25**	-1,22
RBL1	**-1,76**	1,21	**-2,22**	-1,38
CDC2	-1,24	**1,57**	**-2,04**	-1,49
MCM5	**-1,51**	1,17	**-2,00**	**-2,59**
MAD2L1	-1,11	1,19	**-1,92**	**-1,51**
HERC5	1,25	**2,10**	**-1,91**	1,35
CDKN2B	**-1,59**	**-1,66**	**-1,91**	-1,31
MKI67	**-2,21**	**1,82**	**-1,86**	**-2,98**
CDKN2A	1,01	-1,22	**-1,81**	1,05
GTSE1	-1,37	1,42	**-1,77**	**-2,06**
PCNA	**-1,64**	1,42	**-1,76**	**-2,16**
CHEK1	**-1,64**	1,12	**-1,68**	-1,47
CDC20	-1,20	1,46	**-1,62**	-1,25
KNTC1	**-1,76**	1,39	**-1,57**	**-1,67**
CCNB2	-1,13	1,13	**-1,53**	**-1,71**
GAPDH [a]	*1,38*	*-1,23*	***-1,52***	-1,02
RAD1	-1,05	**2,27**	**-1,50**	**-1,77**
MCM3	1,28	1,23	-1,46	-1,31
MRE11A	**-1,79**	**2,04**	-1,42	**-1,64**
BCL2	**-1,58**	**2,15**	-1,35	-1,23
CKS2	-1,26	**2,15**	-1,31	**-1,57**
CKS1B	1,03	**1,55**	-1,29	-1,07
RPA3	-1,19	1,29	-1,22	**-1,60**
RBBP8	-1,08	1,39	-1,21	-1,30
CHEK2	**-1,52**	-1,08	-1,19	**-1,65**
KPNA2	-1,47	1,29	-1,18	-1,07
CDK6	-1,47	1,49	-1,17	-1,07
ACTB [a]	*-1,42*	*-1,19*	*-1,17*	1,19
SKP2	**-1,52**	1,20	-1,17	**-1,53**
ABL1	-1,18	1,27	-1,10	-1,15
CCNF	**-1,68**	1,49	-1,09	-1,30
CCNB1	1,02	**1,53**	-1,07	**-1,60**
HPRT1 [a]	*1,14*	*1,10*	*-1,06*	**-1,65**
CDK4	-1,02	-1,06	-1,05	-1,02
NBN	**-1,55**	1,25	-1,01	**-2,47**
CCNE1	**-2,23**	**-3,24**	-1,00	-1,34
MCM4	**-2,15**	**1,99**	1,03	**-2,47**
RAD17	1,01	1,29	1,06	-1,44
ATM	**-1,74**	**2,04**	1,09	-1,31
CUL3	-1,39	1,09	1,11	-1,17
CCNH	1,00	1,26	1,11	-1,16
RPL13A [a]	*1,05*	*1,20*	*1,12*	1,19
GADD45A	-1,48	1,32	1,13	1,03
CDKN1B	**-1,99**	**1,83**	1,19	**-2,05**
RB1	-1,00	-1,01	1,19	1,05
CDC16	-1,19	1,13	1,19	-1,09
BCCIP	-1,16	1,30	1,20	-1,20
TP53	-1,46	1,13	1,22	1,24
MAD2L2	-1,08	**1,73**	1,24	-1,24
HUS1	**-1,85**	**1,82**	1,25	**-1,54**
CCND1	-1,49	-1,22	1,25	-1,43
UBE1	1,21	-1,16	1,26	1,21
CDK5RAP1	-1,36	-1,13	1,26	**-1,53**
CCNT1	**-1,74**	1,18	1,26	-1,34
E2F4	-1,07	-1,16	1,27	-1,13
CDK7	-1,05	**1,50**	1,27	1,02
CUL2	-1,29	1,02	1,29	-1,14
CDC34	**1,77**	1,39	1,31	**-2,06**
BAX	-1,28	**1,74**	1,33	-1,22
RAD9A	-1,25	1,02	1,35	-1,42
CCNC	-1,08	1,01	1,35	1,44
CCNG1	1,09	-1,09	1,36	**1,61**
CUL1	1,03	1,02	1,37	-1,08
CCNG2	-1,37	-1,11	1,39	**-1,87**
GTF2H1	**1,80**	**1,71**	1,40	**-1,62**
MNAT1	-1,20	1,32	1,44	-1,05
ANAPC4	1,11	-1,01	1,48	1,02
ATR	-1,11	1,37	1,49	-1,30
CDK5R1	**-1,61**	1,22	1,49	1,21
CCNT2	-1,15	1,22	**1,58**	**-1,63**
CDKN1A	1,02	**1,52**	**1,58**	1,29
SUMO1	-1,48	1,26	**1,63**	-1,40
SERTAD1	1,04	1,01	**1,66**	-1,09
CDK8	-1,17	1,05	**1,69**	1,10
B2M [a]	*-1,17*	*1,11*	***1,69***	1,19
DNM2	-1,44	**1,67**	**1,71**	-1,26
ANAPC2	-1,25	-1,01	**1,71**	**-2,11**
TFDP1	**-1,81**	1,22	**2,01**	**-1,63**
TFDP2	-1,07	1,34	**2,16**	1,15
RBL2	-1,30	1,02	**2,71**	1,13

[a] Five control/housekeeping genes are included in the RT *Profiler* PCR array: B2M, HPRT1, RPL13A, GAPDH and ACTB.
[b] Fold-change in the presence of Dox versus in the absence of Dox (6 days) is presented in ascending order for the H1.2 shRNA cell line.

Table 4. Cell cycle-related genes with altered expression in the H1 knocked-down cell lines.

2.2- Specific participation of H1 subtypes in nuclear processes.

2.2.1- Role of H1 variants in hormone responsive promoters.

Because of the scientific interests of our laboratory, we have extensively studied the consequences of H1 depletion on gene expression, not only on basal gene expression but also, in response to an hormonal stimuli such as progesterone and, in particular promoters (MMTV and 11beta-HSD1) currently used by the group as models to study the interplay between chromatin and promoter activity (See Introduction).

2.2.1.a- Gene expression changes upon hormone induction in H1 knocked-down cell lines.

Microarrays experiments comparing all the knock-down cell lines revealed a different set of genes altered in each case. Mainly we can find two different behaviours. On one hand, we have identified genes where a synergy between hormone treatment and shRNA expression was found, meaning that H1.2 inhibition altered the hormone responsiveness of these genes. The majority of these genes have lost their ability to respond to R5020 in the knocked down H1.2 cell line. On the other hand there are also a heterogenous group of genes in which the hormone is modulating the effect of the shRNA.

2.2.1.b- Role of H1 on the progesterone response of the MMTV promoter.

Our laboratory showed some years ago, that histone H1 represses basal transcription and activation by either the progesterone receptor (PR), or the Nuclear Factor 1 (NF1) individually in the MMTV promoter, and in particular in the nucleosome B (nucB) region, but enhances transactivation in the simultaneous presence of both factors. In the model proposed, it is suggested that binding of PR to the exposed HREs in the nucB recruits or activates a kinase that phosphorylates histone H1. This leaves the promoter in a last step once ATP-dependant chromatin remodeling complexes and NF1 open and stabilize the nucleosomal structure to enable efficient transcription initiation.

By chromatin immunoprecipitation studies (ChIP) in T47D-MTVL cell line we showed that H1 is present in the nucleosome B (nucB) of the MMTV promoter and leaves the promoter after 30 min of hormone induction. On the contrary, PR is strongly recruited upon 15 min of R5020 treatment (Figure 24A).

In order to explore the status of H1 during the remodeling process before leaving, we performed ChIP experiments using antibodies against phospho-H1 (Upstate) and acetylation of the K26 residue of H1.4. Interestingly, there was an increase in both acetylation and phosphorylation, at 15 minutes of hormone induction, just before leaving from the promoter (Fig.24B).

Previously, we have shown that phospho-H1 (Upstate) antibody recognizes mainly H1.4 and the acetylated K26 antibody was made from a specific H1.4 peptide. According to this, we can speculate about the specific involvement of H1.4 in the regulation of the MMTV but in fact, we can not exclude that other phosphorylated variants were also recognized by the antibody or that other subtypes were also acetylated and recognized by the antibody, as this modification has been only described for H1.4 so far. Thus, we can conclude that phosphorylation and acetylation may be playing a role during the activation process of the MMTV promoter as other histone modifications identified by other members in the group, for instance phosphorylation of S10 in H3 (Vicent et al. 2006).

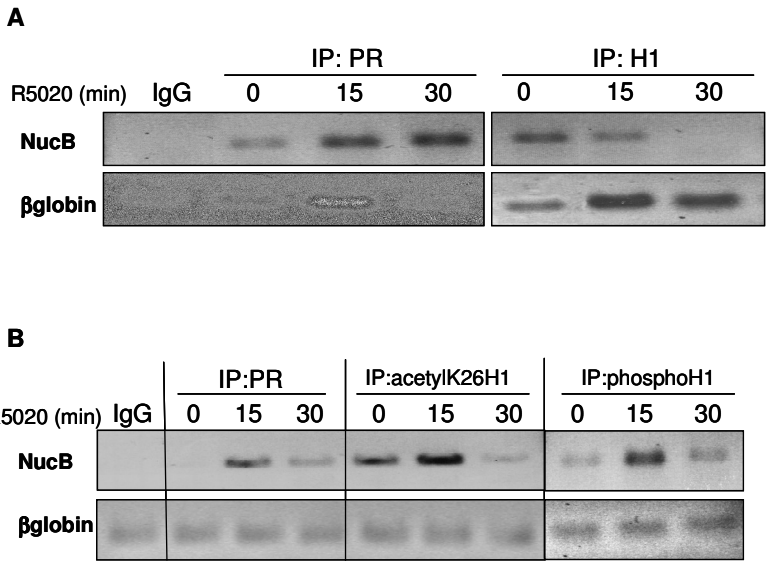


Figure 24. Displacement of linker histone H1 from the MMTV promoter upon hormone induction.

(A) ChIP experiment against total H1 and PR after indicated times of R5020 10^{-8} M (progesterone analog) treatment. Total H1 AE-4 (Upstate) and PR H-190 (Santa Cruz) antibodies were used. Amplification of nucB region was tested by PCR. Bglobin promoter was used as control gene that does not change with hormone induction. IgG means non-related antibody.

(B) Acetylated and phosphorylated status of H1 was tested by ChIP using home-made acK26 antibody (Dr. Reinberg's lab) and phospho-H1 (Upstate) antibody. IgG means non-related antibody.

2.2.1.c- Differential location of H1 variants in 11β-HSD2 and MMTV promoters.

HA-tagged H1 variant-expressing cell lines have been used mainly to investigate the role of H1 subtypes in the hormone activation process of two promoters: MMTV and 11β-HSD2. In both cases, the mechanism of activation is being characterized in detail by the group. The objective is to explore which is the H1 subtype(s) involved in the activation process in the case

that there is a variant-specificity and in which step is important. For this, we performed ChIP experiments against each HA-tagged H1 variant using an anti-HA antibody (Abcam) and looking at the nucB region of the MMTV and the distal region of 11beta-HSD, both regions have been described to recruit PR and were remodeled upon hormone induction.

The figure 25A showed our control for the hormone induction process. After 5 min of R5020 treatment, PR is efficiently recruited in nucB region of the MMTV and in distal region of 11β-HSD2 as it has been described by other members of the group previously. β-globin gene is not affected by hormone treatment and it was used as a control of the immunoprecipitated material loaded, in consequence the PCR was much less efficient and the number of cycles was increased. The PR recruitment was done as control for each H1-HA variant cell line with the same result every time, although it is shown only once.

Panels in figure 25B showed the ChIP results obtained for the each of the H1-HA expressing cell lines after hormone induction. According to these results, in the nucB region of the MMTV all the variants were present at point 0 and displaced after 30 min of R5020 except H1.0 whose presence was similar in all the times analyzed. Interestingly, this behavior does not coincide in the case of the 11β-HSD distal region. From the variants analyzed only H1.3 disappeared clearly from the promoter upon hormone induction while H1.0 and H1.5 displacement was not very clear and should be quantified by RT-qPCR before any conclusion.

Surprisingly, βglobin amplification was highly efficient in the case of H1.4-HA, suggesting a possible specific location of this variant to the tested region of the βglobin gene. We have also introduced in this analysis a H1.4 mutant HA expressing cell line. This cell line expresses the variant H1.4 with a point mutation in the residue 26. The Lys26, involved in the recruitment of SirT1 and PRC2 complex, was changed to Ala (courtesy of Dr. Reinberg's lab). This mutant variant is also located to the βglobin promoter although the signal for nucB region seems not to be specific if we compared the non-related antibody signal (IgG) with the rest of the time points.

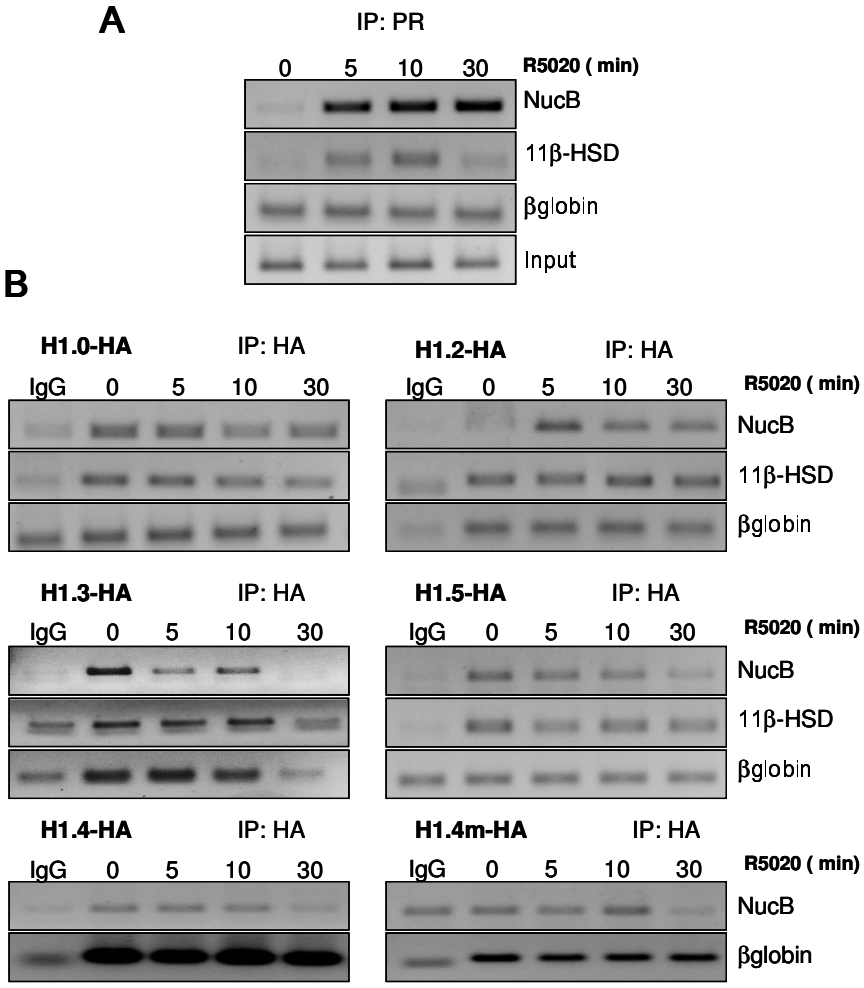


Figure 25. Differential location of H1 variants in two different hormone responsive promoters: MMTV and 11β-HSD2.

(A) PR recruitment upon hormone induction in nucB of MMTV and distal region of 11β-HSD2. ChIP experiments performed in H1.2-HA expressing cell line using PR H-190 (Santa Cruz antibody). Similar results were obtained for the rest of the H1-HA variant cell lines. IgG means non-related antibody.

(B) H1-HA subtype location in the two promoters analyzed after hormone induction. Immunoprecipitation was done by HA antibody (Abcam). IgG means non-related antibody.

2.2.1.d- Distribution of H1 subtypes in hormone-responsive promoters: ChIP-on-chip tiling arrays.

At the same time as we investigated the specific recruitment of H1 variants in two hormone-responsive promoter models by ChIP, we prepared sample from each HA-tagged H1 variant expressing cell line treated or not with R5020 for 30 min, to perform ChIP-on-chip experiments. This will allow us to study whether upon a stimulus such as hormone activation there is any established pattern of changes in H1 variants for controlling specific promoters/gene expression. The main objective is to clarify if a particular variant plays a global role, or different variants are involved in different promoters.

A custom tiling array was designed in the laboratory to study in detail changes in histone modifications, transcription factors and so on. It covers regions from -8.0 Kb upstream to +2.0 Kb downstream of the transcriptional start sites of 42 selected genes, 40 progesterone-responsive and 2 control promoters. The sequences were tiled with 60 mer in situ-synthesized oligonucleotides with 20 bp periodicity (40 bp overlap), generating an average of 497 probes per gene. Samples were prepared and inmunoprecipitated as regular conditions for ChIP. In a last step, immunoprecipitated material was amplified and tested by PCR in the nucB region of the MMTV just before hybridization protocol (Figure 26).

The ChIP-on-ChIP tiling arrays done using our customized high resolution platform revealed

2.2.2- Identification of interacting partners specific for the different H1 variants.

2.2.2.a- Role of H1.4 variant in the formation of facultative heterochromatin.

Recently, it has been described that H1.4 can be methylated and acetylated to Lysine 26 (K26) (Kuzmichez et al. 2003, Vaquero et al. 2004) by PRC2 complexes. PRC2 complex is also able to deacetylate and methylate Lysine 27 (K27) of histone H3. Dr. Reinberg's laboratory showed an interaction *in vivo* between H1.4 and SirT1, a deacetylase enzyme involved in heterochromatinization. These results provide a new piece of information about the specific role of one of the H1 variants in a transcriptional regulation process such as promoter silencing. H1 is a key component in the open chromatin process and has to be post-translationally modified in order to regulate the access to transcriptional machinery. We had the opportunity to participate in the project and in particular, in the characterization of the protein complex able to deacetylase and methylate K26 of H1.4.

Inmunoprecipitation experiments overexpressing transiently H1.4-HA in 293F cells revealed that H1.4 was able to pull-down Ezh2 and SirT1 (Figure 28A), both members of the PRC2 complex, pointing to the fact that they form together a complex. This interaction was disrupted when we mutated the Lysine 26 to an Alanine (Figure 28B). This means that residue K26 that could be deacetylated and methylated, is important for the recruitment of complexes with histone modification activities such as PRC2.

In addition to this, it has been described that Akt phosphorylates Ezh2 and this could be regulating the substrate specificity of the complex. We tested whether inhibition of Akt by an specific inhibitor such as Ly could be altering the complex composition or its substrate specificity. When Ezh2 was phosphorylated the interaction with H1 was disrupted and SirT1 was not present in the complex (Figure 28C), consequently di/tri methylation of K27 of H3 was decreased (Figure 28D). These results open a new view in the way PRC complexes are regulated and their substrate specificity.

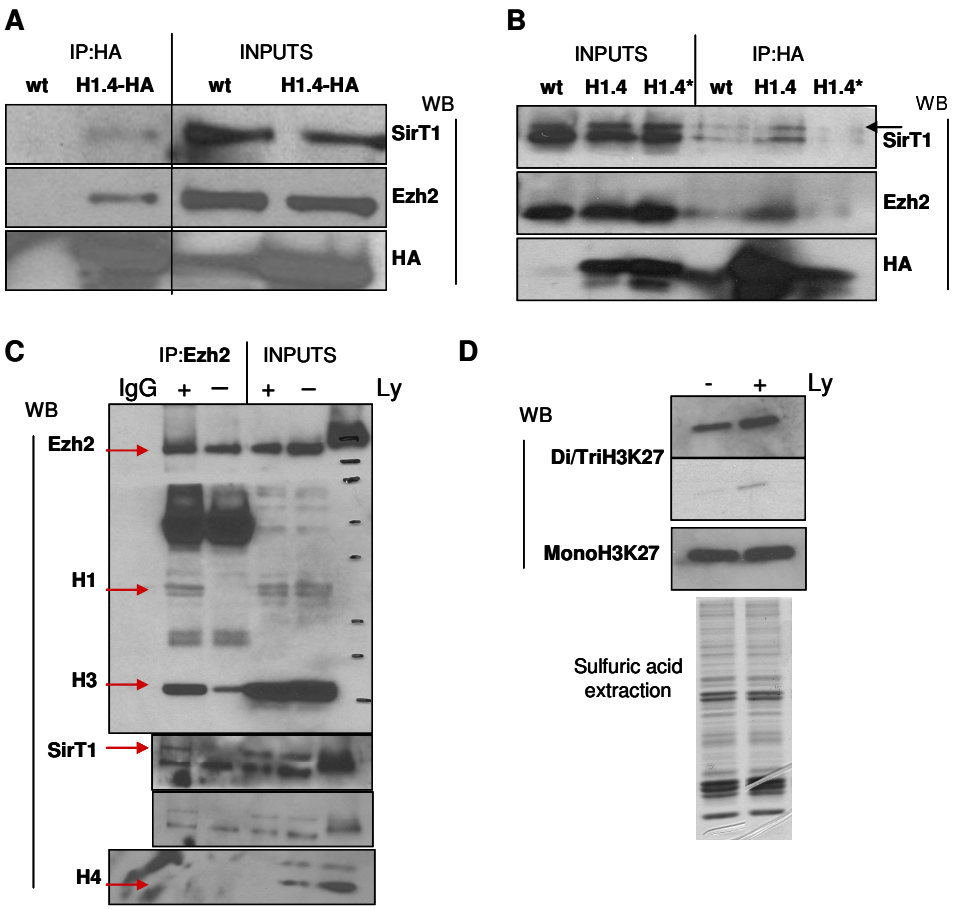


Figure 28. Involvement of H1.4 in PRC2 complex and new insights in the regulation mechanism of Ezh2.

(A) Interaction between H1.4 and two members of the PRC2 complex. Nuclear extracts from cells expressing H1.4-HA were immunoprecipitated against HA tag. Western-blot analysis was performed with specific home-made antibodies against Ezh2 and Sirt1.

(B) Immnuprecipitation assay performed in the same conditions as in (A) but this time comparing the ability of interaction of H1.4 when residue K26 was mutated to Alanine (A). Symbol * means expression of the mutated H1.4 protein.

(C) Inmunoprecipitation of Ezh2 from 293F cells treated or not with Ly drug (inhibitor of Akt) for 6h before preparation of nuclear extracts.

(D) Histone purification from 293F cells treated for 6h with Ly inhibitor. Western-blot analysis was performed against the indicated antibodies. Coomassie staining is shown as loading control.

3. Attachment: ongoing experiments.

3.1- Proteomic identification of partners co-immunoprecipitating with H1 subtypes.

The generation of HA-tagged H1 variants vectors and cell lines has open the possibility to study in detail partners of H1 that could be variant-specific and may be involved in their specific roles. We have set up in our laboratory the conditions for immunoprecipitating the different HA-H1 subtypes from 293T transiently transfected or from stable expressing T47D-MTVL cells. Beads attached to anti HA antibody were used to perform affinity purification of H1 and interacting partners.

The immunoprecipitation methodology is working and ready to start with the proteomics analysis. Eluted sample will be resolved in SDS-PAGE followed by silver staining. Discrete bands, appearing respect to the mock purification (HA-expressing cell line, not attached to H1), will be selected and identified by mass-spectometry after peptide digestion.

3.2- Specificity of H1 variants distribution in the nuclei and genome.

3.2.1- Nuclear distribution of H1 variants by immunofluorescence.

Differential nuclear distribution of H1 variants has been a controversial topic in the field. There are several publications that do not coincide exactly in the registered observations. While most of them agree that H1.2 seems to be uniformely distributed in the nuclei, the presence of H1.3 and H1.4 in open or closed chromatin is not yet clear. To clarify this topic we investigated whether H1 variants are distributed differently in the nuclei of human cells and their degree of co-localization with markers for euchromatin and hetrochromatin. For that purpose, we used our stable expressing H1-HA cell lines and the antibodies we have generated, together with the anti-HA to compare different patterns of staining among different H1 variants. Moreover, we wanted to relate possible differential patterns of nuclear distribution to functional regions in the genome. For this, we co-stained with markers for transcriptionally active regions (euchromatin) such as phoRNA pol II or for silenced chromatin (heterochromatin) such as HP1gamma.

3.2.2- Genome-wide distribution of H1 variants: ChIP-on-chip.

As we have commented previously, HA-tagged H1 variant-expressing cell lines are a powerful tool to investigate on ChIP-on-ChIP experiments how H1 variants are distributed in the genome, whether any position is occupied by a particular variant preferentially or all variants are randomly distributed and exchangeable. After the encouraging results obtained in

the small high resolution platform previously described, next step in our research will be to move to a larger platform containing all human promoters in order to extend the knowledge about specific roles of H1 variants as transcriptional regulators.

DISCUSSION

Histone H1 family of proteins are found associated with linker DNA regions connecting adjacent nucleosomes in eukaryotic chromatin. Binding of linker histones stabilizes and induces condensation of the chromatin filament into a 30nm fiber (Van Holde, 1988). In contrast to core histones, linker histones exhibit a higher heterogeneity and show much less evolutionary conservation (Cole RD, 1987). This variability may indicate a relatively less critical contribution to chromatin function but does not explain why so many subtypes have appeared during evolution in mammals. Two main features are conserved during evolution, suggesting a crucial role for these proteins. First, linker H1 proteins present a three-domains structure. The globular domain is the most conserved part of the protein, not only among subtypes but also during evolution. Tail domains are divergent in their lengths and sequences and constitute the most distinctive features of each variant (Wolfe et al, 1987). And second, genes encoding H1 proteins are differentially expressed along development and in cell types (Khochbin and Wolfe, 1994). These observations suggest that H1 variants have to accomplish specialized functions.

In order to elucidate the differential functionality of histone H1 diversity we have developed a set of powerful tools to study in detail differences of somatic H1 subtypes in cell proliferation, their influence on transcriptional regulation and the mechanisms by which H1 either stimulates or represses transcription of specific genes.

On one hand, we have produced a novel set of variant-specific antibodies very useful to detect specifically each subtype by western–blot techniques. In addition, a collaboration project with Abcam Ltd. (Cambridge, UK) will allow us to have at our disposal new antibodies against specific modifications of somatic H1 variants, an important topic due to the increasing interest of the research field in the role of H1 as transcriptional regulator.

On the other hand, we have generated stable cell lines derived from breast cancer T47D where the different H1 variants have been knocked down by inducible expression of specific shRNAs. These cell lines provide an opportunity to explore the in vivo influence of H1 variants on chromatin organization, transcriptional repertoire and growth behavior of the cell. The constitutive shRNA expression system was not a successful approach due to the difficulties for maintaining the inhibition along the time for some H1 shRNAs. The lost of inhibition suggests a role of a particular subtype in the regulation of genes related to survival and the necessity of the cell to adapt to a disadvantageous environment. We have solved the problem setting up an inducible shRNA expression system that avoids long exposures to the shRNA and as a result, the development of adaptative or compensatory mechanisms.

Finally, we have also generated stable cell lines expressing HA-tagged H1 variants in order to explore the specific recruitment of the different subtypes in particular promoters by ChIP and ChIP-on-chip methodology.

H1.2 and H1.4 are indispensable for cell proliferation.

Cell cycle studies in the inducible knocked-down cell lines have shown a G1 arrest and a cell death phenotype upon depletion of H1.2 and H1.4, respectively. This is the first time of an indication of two essential variants for cell cycle progression in a human breast cancer cell line such as T47D. Interestingly, the clones constitutively expressing the shRNAs for H1.2 and H1.4 lost correspondent inhibition. This suggests a negative selection for those cells with high level of H1.2 and H1.4 inhibition. In addition, it has not been yet found a cell line from human tissue cultures that does not express H1.2 and H1.4 (Meergans et al. 1997).

In the case of H1.4 depletion, we were not able to fully understand the phenotype shown. Apoptosis studies performed did not reveal any indication of an activated cell death program and the phenotype seems to be exclusive of T47D and MCF10A cells. The first cell line is a human breast cancer cell line characterized by mutations in caspase-3 and p53, main transducers of the apoptosis pathway and in consequence, highly resistant to undergo this process. Unfortunately, we can not exclude secondary effects of shRNA as we did not success to find another shRNA unable to inhibit this variant. All these observations indicate that we have to be cautious with the result and further studies need to be considered such as the re-introduction of H1.4 variant resistant to the shRNA in order to confirm the specificity of the phenotype.

However, in the case of H1.2 knocked down cell lines we have demonstrated that the G1-arrest is specific and exclusive of the lack of H1.2. All the shRNAs sequences designed for all the H1 variants were selected for low homology with other genomic regions to avoid secondary effects. Possible toxic effects of doxicycline were controlled by the controlsh and the other knocked-down cell lines that did not show similar effects. Although a second working shRNAs was not found, the complementation of the phenotype is almost complete with the expression of a shRNA-resistant H1.2 subtype, not only at the level of cell cycle progression but also in chromatin organization and gene expression. In addition, other cell types tested such as MCF-7 and MCF10A showed difficulties along cell cycle progression. Cell cycle studies did not show a clear G1 arrest although we have described an increase in subG1 peak, an indirect measurement of apoptosis. We can argue that H1.2 is important for life span of the cell and its

depletion triggers a signal of wrong status that ends up with a cell cycle stop response mediated by different mechanisms, depending on the cell type.

H1.2 depletion causes alterations in chromatin structure.

We have also investigated possible alterations in chromatin structure in the different knocked-down cell lines and we have observed an average 8-bp reduction in nucleosome repeat length (NRL) in the H1.2 depleted cells. Surprisingly, a reduction of the 20% of the total H1 content achieved in this cell line alters specifically the NRL. This means that the amount of linker histones per nucleosome is reduced, charge homeostasis tends to be restored by a reduction in nucleosome spacing, creating a change in chromatin conformation from a zig-zag arrangement of nucleosomes and linker DNA to a more open and randomly folded beads-on-a-string form.

In the mouse, deletion of one or two H1 subtypes results in a compensatory upregulation of other subtypes, resulting in the normal level of H1/nucleosome, and no apparent phenotype (Sirokin et al 1995; Fan et al 2001, 203). Knocking out additional subtypes creates a situation in which the upregulation process fails to fully compensate and a reduction in NRL occurs. Authors claimed that in triple-H1 KO ES cells (50% of reduction in total H1), the electrostatic effect of this reduction can be compensated by the reduction in NRL together with other mechanisms such as changes in core histone modifications. As far as we have explored, no global changes in core histone modification were observed in our H1.2 knocked-down cell line. Altogether suggests that our inducible H1.2 depletion model system is not able to fully compensate the charge homeostasis and this could be directly related to the phenotype of cell cycle arrest observed.

However, it is still not clear how a 20% reduction of the total content of H1 causes a striking reduction in NRL. Eukaryotes exhibit a wide range of NRLs not only between species, but also between cell types within an organism (van Holde 1989). While neuronal chromatin had a NRL of 162 bp and an H1 ratio of 0.45 molecules per nucleosomes, the values for glia were 201 bp and 1.04, respectively. One possible explanation for our observation could be that reductions of total H1 content are not tolerated in the same way in all cell types, pointing to the existence of a minimal H1 content threshold cell type dependent. Despite of this, similar H1 variant reductions in this cell line do not cause alterations in nucleosome spacing and cell proliferation pointing to a specific role of H1.2. It may be that H1.2 affects the binding and distribution of the rest of subtypes.

Different set of genes are affected by specific H1 variant depletion.

Gene expression profiles in H1.2 knocked-down cell line show a low number of genes affected by the shRNA. Moreover, the majority of these genes had reduced expression in the knocked-down cell lines. These results might be viewed as surprising, because most of the *in vitro* studies have suggested that H1 linker histones may function primarily in causing repression of gene transcription. However, very similar results were obtained in microarray studies of H1 mutant yeast and mouse H1t mutant spermatocytes. The removal of linker histones does not affect the vast majority of genes and instead causes changes in expression of a small group of genes. Thus H1 may have both positive and negative effects on gene transcription but the mechanisms remain to be elucidated.

Although genes with altered expression can be distributed into many functional groups as figure 1 summarizes, the main group of affected genes are related to cell cycle, DNA replication and DNA repair. Genes such as cdc2, cdkn3, ccnb2, cdc6, cdc45l and so on, are directly involved in the progression of the cell cycle. Repression of some of these genes may correlate to the G1-arrest described for the H1.2 knocked-down cell line. More accurate studies to define a set of cell cycle related genes altered by H1.2 depletion that could explain the phenotype has been performed and results will be discuss below.

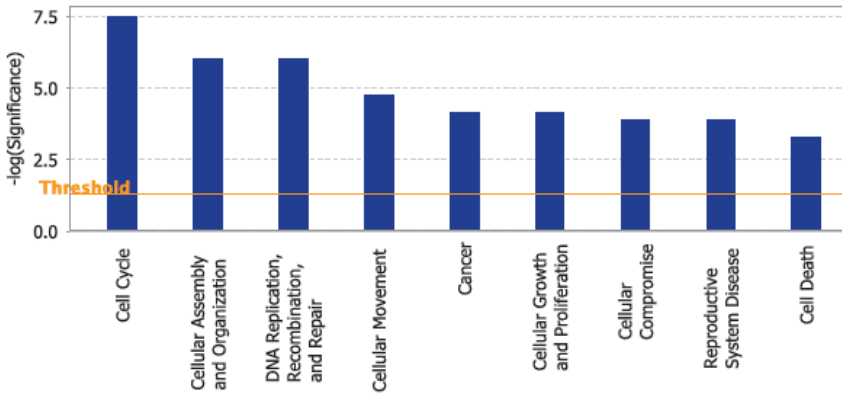


Figure 1. Representation of a functional distribution of the genes affected in the microarray analysis.

Expression of cell cycle-related genes is altered in H1.2-depleted cells

Microarray results in the H1.2 knocked-down cell line suggest a pattern of gene expression altered that could explain the arrest phenotype. The gene expression profile shown may be reflecting only the status of the majority of the population (arrested in G1 as PI studies indicate) but we considered unlikely this possibility if we realized that all the populations of

cells in the experiment were already synchronized in the same phase. Thus, differences in gene expression should be due to H1.2 depletion.

In order to clarify this point we further explored the relationship between H1.2 depletion and the cell cycle gene expression profile by qRT- PCR using the SuperArray cell cycle specific platform. Controlsh and H1.2 KD cell lines treated or not with Dox were serum-deprivated for 48h and arrested in G1 phase. Serum addition was a strong signal to move the cells through cell cycle except in the case of H1.2 depletion. In those conditions we identified a set of genes whose expression is altered that could be responsible for the cell cycle phenotype (Figure 2).

G1-phase and G1/S transition:	CDK2, CDKN3
S-phase and DNA replication:	MCM2, MCM3, MCM4, MCM5, RPA3, PCNA
G2 phase and G2/M transition:	BIRC5, CCNB1, CDKN3, CKS1B, CKS2, GTSE1, KPNA2, CCNT2, CDK7, ANAPC4
M phase:	CCNB2, CDC2, CDC20, MRE11A
Cell cycle checkpoint:	CDKN2A, CDKN2B, CDKN3, CHEK1, KNTC1, MAD2L1,RAD1, RBBBP8, TP53, BRCA1, CCNG2, CDC2, CDK2, CDKN1A,
Negative regulators of cell cycle:	BRCA1, RBL1, RBL2, CDKN2B, TP53

Figure 2. Summary of the results obtained by PCR Arrays in the H1.2 depleted cell line. Genes have been classified according to its functional group. Colour blue indicates repression and red activation.

Cell cycle progression is a complex process tightly regulated by multiple signalling pathways and regulatory networks that govern activation and suppression of genes controlling proliferation.

In proliferating cells, the cell cycle consists of four phases. G1-phase is the interval between mitosis and DNA replication that is characterizes by cell growth. The transition that occurs at the restriction point (R) in G1 commits the cell to the proliferative cycle. If the conditions are not optimal, the cell exits the cell cycle and enters G0, a non-proliferative phase during differentiation and apoptosis occur. Replication of DNA takes place during the S (synthesis) phase, which is followed by G2 during which growth and preparation for cell division occurs. Mitosis, as production of two daughter cells, occurs in M-phase.

Passage through the four phases is regulated by a family of cyclins that act as regulatory subunits for cyclin-dependent kinases (CDKs). The activity of the various cyclin/CDK complexes that regulate progression of the cell cycle is controlled by the synthesis of the appropriate cyclins during a specific phase of the process. The cyclin/CDK complex is then activated by

sequential phosphorylation and dephosphorylation of key residues, located principally on the CDK subunits.

The cyclin/CDK complex of early G1 is either cdk2, cdk4 or cdk6 bound to a cyclin D isoform. This complex can be inhibited by several proteins such as p21, p27, p15 or p16. In this circumstances, retinoblastoma protein (Rb) is in a state of low phosphorylation and is tightly bound to the transcription factor E2F, inhibiting its activity. In order to pass the R point, cyclinD/CDK complex is activated and Rb becomes phosphorylated. Cyclin E/CDK2 accumulates during late G phase, completes the phosphorylation of Rb and triggers the passage into S phase. Phosphorylated Rb dissociates from E2F, which initiates the transcription of key S-phase-promoting genes including some that are required for DNA replication The entire genome is replicated during S-phase. The synthesis and accumulation of cyclin b/cdc2 also begins during S-phase, but the complex is inactive by phosphorylation of specific residues. Cyclin A/cdk2 accumulates during S phase and its activation triggers the transition to G2, a phase characterized by the accumulation of cyclinB/cdc2 (CDK1). As cells approach M-phase, the phosphatase cdc25 is activated by phosphorylation which in turns activates cdc2 and drives the cell into mitosis.

Cell cycle-related genes altered in the H1.2KD cell line can be classified according to their role in different phases of the cell cycle as it is summarized in figure 2. To try to find a candidate(s) for the G1-arrest phenotype is not easy. Cdc2 is one of the genes more consistently inhibited in the different experiments. Although classically it has been considered as a G2/M regulator, new studies indicate that it is also playing a role in G1/S transition (Martin et al. 2005; Aleem et al 2005). Cdc2 is able to compensate the loss of CDK2 function in knockout mice and it has been described that cyclin E binds to and activates Cdc2. As it has been explained cyclin E/cdk complex is also participating of the phosphorylation of Rb leading to activation of E2F-mediated transcription. Interestingly, we were not able to see changes in the protein level of Cdc2 but the levels of phosphorylated Rb are decreased in the H1.2 depleted cells. The cdc2 level has been checked only once while phospho-Rb was reproducible several times. Further efforts in this direction should be considered.

Other possible candidate would be cdkn1a (p21), also consistently activated, although we do not have detected an increase in the protein level yet. It has been recently suggested that alteration in histone H1 metabolism (reduced level of chromatin-bound endogenous H1) may be involved in a cellular senescence-inducing mechanism (Funayama et al. 2006). Cellular senescence is a tumor-suppressing mechanism that is accompanied by characteristic chromatin condensation called senesce-associated heterochromatic foci (SAHFs). Ishikawa

group found that H1 is lost from senescent cells and substituted by HMGA2 protein. Moreover, senescent cells showed alterations in expression of genes, in particular a decreased in phospho-Rb and cyclin A and increased p21 and phospho-p53. It remained to be clarified whether histone H1 loss plays a causative role in senescence induction or is only an effect of the process. Some of these effects are also seen in our cell model such as a reduction in the levels of phosphor-Rb, a increase in p21 gene expression and a clear reduced of the H1.2-bound chromatin (as NRL experiments demonstrate). Further studies to investigate whether H1.2 depleted cell lines show a senescent phenotype will be done.

In addition to this, we can not exclude that replication process may be impaired. MCM genes are also highly repressed. The complex processes of DNA replication are under many levels of cellular control. In higher eukaryotes, DNA synthesis is initiated at discreet loci, called origins, which are recognised by the multiprotein origin recognition complex (ORC). In G1 phase, origins are licensed by the action of Cdc6 and Cdt1, which trigger the association of replication initiation proteins such as MCMs, with ORC. These proteins are probably the helicase complex in charge of separating the two strands of DNA. PCNA is also involved in this process by topologically encircling the DNA at the site of synthesis.

Until now discussion has been focused on the possible role of H1.2 as transcriptional regulator of certain key genes involved in cell cycle progression in order to explain the phenotype. However, we have to consider the possibility that H1.2 is also participating directly in some important cellular processes. For instance, H1.2 has been identified as a cytochrome c-releasing factor that appears in the cytoplasm after X-ray irradiation and induces cytochrome c release from isolated mitochondria (Konishi et al. 2003). These results indicate that H1.2 may be playing a role in transmitting apoptotic signals from the nucleus to the mitochondria following DNA double-strands breaks. Moreover, chromatin decondensation in S-phase involves recruitment of Cdk2 by Cdc45 and H1 phosphorylation, suggesting also a participation of H1 in DNA progression and fork progression (Alexandrow and Hamlin 2005). Every time more frequently papers appear describing additional and specific functions of H1 variants but still there are many questions open. So far we do not have any preliminary study suggesting that H1.2 may be involved in any cellular process such as DNA replication and this is the cause of the phenotype, but future directions will consider this possibility.

We currently favour a model according to which H1 variants have two distinct roles, a common and a specific one. Individual H1 proteins are redundant in their ability to compact chromatin globally and to stabilize overall higher order chromatin structures. However, at local chromatin organization, each individual could be regulating a subset of specific genes both in a

negative and positive way and this could have direct consequences in life span depending on the cell type.

Summary.

Organisms such as *Ascolobus*, *Saccaromyces* and *Arabidopsis* lacking H1 have shortened life span, an indication that loss of H1 has severe consequences even in these organisms. In lower eukaryotes, its absence does not affect short-term survival but leads to accumulative defects that ultimately are detrimental to long-term survival. In higher organisms such as mice, reduction of H1 below a certain threshold is not compatible with full embryonic development. In this report we have showed that H1.2, and probably H1.4, are essential for cell cycle progression in several human cell lines. How can linker histones be essential for long term survival? On one hand, it has been described that silencing of H1 increases the overall methylation of the genome. The possibility of methylation accumulating with time and spreading to genes that could be critical for long term survival appears to be unlikely. On the other hand, changes in chromatin conformation arising from the lack of histone H1 appears to be more likely explanation.

Under physiological conditions and in the absence of histone H1, the chromatin fiber has a more extended organization in which the nucleosomes are still in close contact but lack the three-dimensional organization that is characteristic of native chromatin as it seems is happening in the H1.2 depleted cells. DNA is more accessible to a variety of regulatory proteins positively or negatively affecting the expression of specific genes. It could be that control genes of the cell could be incorrectly regulated, and the accumulated effects of incorrect patterns of expression eventually lead to a cessation of growth.

Another possibility could be a direct participation of H1.2, for instance, in the regulation of expression of some of the genes described previously. The presence of this specific variant in some of these promoters could trigger to the recruitment of remodeling chromatin complexes and transcriptional regulators that alter their expression. That is, a specific subtype may be necessary for the recognition of regulators or for activation/repression of a particular promoter under specific circumstances. These key affected genes could be involved in the regulation of other related cell cycle genes, spreading the effect and causing a cell cycle stop. Further experiments based on ChIP methodologies in specific promoters would bring some light to this hypothesis. All these mechanisms are not necessarily mutually exclusive and might operate synergistically.

The chromatin fiber is a metabolically active, dynamic and flexible structure that continuously changes in response to a wide range of biological signals such as transcription, replication, repair and recombination. These processes are associated with nucleosome remodeling and involve reorganization of high order chromatin structures. H1 family bind to nucleosomes, stabilize the condensed high-order chromatin structure, changes the nucleosome repeat length, minimizes nucleosome sliding, modulates the activity of nucleosome remodeling complexes, and has effects on transcriptional activities.

Depletion of H1 variants changes the cellular transcription profile by either increasing or decreasing the expression levels of specific genes. H1 variants can modulate the activity of chromatin binding regulators at the higher-order chromatin level, stabilizing a more compact structure and reducing access to nucleosomes. They can also affect the nucleosome binding of both activators and repressors by acting as steric factors that could either enhance or reduce the ability of regulators to bind to their nucleosomal targets.

In contrast to genetic experiments in mice that point to the redundancy role of H1 proteins, functional differences among H1 variants are now appearing in the literature. H1-variant substitution may suffice to stabilize the condensed chromatin and to adequately regulate most chromatin functions. However, under special circumstances only a specific variant may be fully functional. H1 variants could associate with specific protein partners that target the subtype to a particular site, could be differentially post-translational modified affecting their binding properties and could have different residence time at a specific locus.

According to a dynamic point of view, H1 subtypes function as members of a network of chromatin binding proteins that continuously remodel the nucleosomes and modulate the accessibility and local structure of the chromatin fiber. The equilibrium of the binding and activity of the various components in the network optimizes chromatin structure and function to satisfy the cellular requirements at any given time. Thus, the function of a missing variant may be compensated not only by a functionally redundant variant H1, but perhaps also by other members of the network. Deleterious effects or strong gene expression changes could be minimized. As a result of this, we believe that the inducible shRNA KD cell lines provide a novel system in which cells do not have time to adapt or compensate new circumstances and where can be investigated specific roles of the different H1 subtypes in detail.

CONCLUSIONS

- Inducible shRNA knocked-down cell lines provide a novel and interesting system in which cells do not have time to compensate new circumstances and where can be investigated specific roles of the different H1 subtypes in detail.

- Depletion of H1 subtypes occurs effectively six days after doxicycline addition, without changes in expression of other subtypes.

- H1 knocked-down cells progress differently through the cell cycle upon H1 inhibition and consequently, proliferate at different rates. H1.0 and H1.3-depleted cells proliferate normally, H1.5-depleted cells proliferate slighty slower, H1.2-depleted much slower and cells show a clear G1-phase cell cycle arrest. H1.4 cells stop proliferating and show a necrotic phenotype, with few cells in S-phase.

- The effects described in H1.2 KD are specific for the lack of this variant. Expression of a shRNA-resistant H1.2-HA protein complements the phenotype at the level of the cell cycle, nucleosome spacing and gene expression.

- Microarray experiments have shown that depletion of different H1 variants does not cause global changes in gene expression.

- Different set of genes are up- or down-regulated in the different knocked-down cell lines, pointing to specific roles as transcriptional regulators.

- Alteration of a set of cell cycle-related genes in the H1.2 KD correlates with the G1-arrest phenotype observed in these cells.

- H1.2 depletion causes a decrease in nucleosome spacing in chromatin that is not seen when other variants are depleted.

- A 20% reduction in total H1 content still have global effects on chromatin organization, indicating that this could be due to a specific role of this variant in chromatin structure, at least in this cell type.

- Alteration of chromatin structure or changes on expression of some important genes could be responsible for the cell cycle alteration in H1.2 knocked-down cell line.

- No changes in DNA methylation and global core histone modifications in the H1.2 depleted cell lines have been observed.

- Differential location of H1 variants has been establish for two model promoters in the laboratory.

- H1.4 interacts with members of the PRC2 complex through the residue K26 and participates in the heterochromatinization process.

ABBREVIATIONS

µm	Micrometer
11b-HSD2	11 beta-hydroxysteroid dehydrogenase type 2
aa	Aminoacid
ADP	Adenosine diphosphate
AF	Activation function 1
AR	Androgen receptor
Arg	Arginine
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
ChIP-on-chip	Genome-wide location analysis
CMV	Cytomegalovirus promoter
Da	Dalton
DAPI	4',6'-diamidino-2-phenyliondole,dihydrochloride
DBD	DNA binding domain
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
Dox	Doxicycline
dsDNA	Double stranded DNA
DTT	DL-Dithiothreitol
EDTA	Ethylene diaminetetraacetic acid
ER	Estrogen receptor
ERID	ER interaction domain
ES	Stem cells
Ezh2	Enhancer of zeste homolog 2
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FRAP	Fluorescence recovery after photobleaching
GFP	Green Fluorescent protein
GR	Glucocorticoid receptor
HAT	Histone acetyltransferase
HDAC	Histone deacetyltransferase
HEPES	(2-Hydroxyethyl)-1-piperazineethanesulphonic acid
HIV	Human Immunodeficiency virus
HMG	High mobility group
HP1	Heterochromatin protein 1
HRE	Hormone responsive element
HU	Hydroxyurea
IP	Immunoprecipitation
K	Lysine
Kb	Kilobase pair
KCl	potassium chloride
KD	Knock-down
Kda	Kilodalton
KLH	Keyhole Limpet hemocyanin
KO	Knock-out
LBD	Ligand binding domain

Lys	Lysine
M	Molar
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium
mM	mili Molar
mm	milimeter
MMS	Methyl methane-sulfonate
MMTV	Mouse mammary tumour virus
Mnase	Micrococcal nuclease
MoMuLV	Moloney Murine Leukemia virus
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
Msk	Mitogen and Stress activated kinase
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NF1	Nuclear Factor 1
NLS	Nuclear localization signal
nm	nanometer
NRL	Nucleosome repeat lenght
nucB	Nucleosome B
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium Iodide
PIPES	1,4-Piperazinediethanesulfonic acid
PMSF	Phenylmethylsulfonyl fluoride
PolII	Polymerase II
PolIII	Polymerase III
PR	Progesterone receptor
PRC	Polycom repressive complex
Puro	Puromycine
RAR	Retinoic acid receptor
RedFP	Red fluorescent protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
Rnase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcription PCR
SDS	Sodium-dodecyl-sulphate
SHR	Steroid hormone receptor
shRNA	Short hairpin RNA
SirT1	Sirtuin 1
TE	Tris-EDTA
TR	Tyroid receptor
Tris	Tris(hydroxymethyl)-amino-methane
VDR	Vitamine D receptor
wt	Wild type

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