Linker Histone Subtypes Differ in Their Effect on Nucleosomal Spacing In Vivo

ARTICLE *in* JOURNAL OF MOLECULAR BIOLOGY · MARCH 2012

Impact Factor: 4.33 · DOI: 10.1016/j.jmb.2012.03.007 · Source: PubMed

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doi:10.1016/j.jmb.2012.03.007 J. Mol. Biol. (2012) 419, 183–197

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Linker Histone Subtypes Differ in Their Effect on Nucleosomal Spacing *In Vivo*

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Received 2 December 2011; received in revised form 17 February 2012; accepted 12 March 2012
Available online 21 March 2012

Edited by J. O. Thomas

Keywords:
linker histone;
H1;
Xenopus oocytes;
chromatin structure;
nucleosomal repeat length

Linker histone H1 is located on the surface of the nucleosome where it interacts with the linker DNA region and stabilizes the 30-nm chromatin fiber. Vertebrates have several different, relatively conserved subtypes of H1; however, the functional reason for this is unclear. We have previously shown that H1 can be reconstituted in *Xenopus* oocytes, cells that lack somatic H1, by cytosolic mRNA injection and incorporated into *in vivo* assembled chromatin. Using this assay, we have expressed individual H1 subtypes in the oocytes to study their effect on chromatin structure using nucleosomal repeat length (NRL) as readout. We have compared chicken differentiation-specific histone H5, *Xenopus* differentiation-specific xH1^0 and the somatic variant xH1A as well as the ubiquitously expressed human somatic subtypes hH1.2, hH1.3, hH1.4 and hH1.5. This shows that all subtypes, except for human H1.5, result in a saturable increase in NRL. hH1.4 results in an increase of approximately 13–20 bp as does xH1^0 and xH1A. chH5 gives rise to the same or slightly longer increase compared to hH1.4. Interestingly, both hH1.2 and hH1.3 show a less extensive increase of only 4.5–7 bp in the NRL, thus yielding the shortest increase of the studied subtypes. We show for the first time in an *in vivo* system lacking H1 background that ubiquitously expressed and redundant H1 subtypes that coexist in most types of cells of higher eukaryotes differ in their effects on the nucleosomal spacing *in vivo*. This suggests that H1 subtypes have different roles in the organization and functioning of the chromatin fiber.

Introduction

The genomic DNA of all eukaryotes is organized in chromatin. The packaging unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped with 1.65 turns around an octamer of core histone proteins and additional interconnecting linker DNA.[1] Eukaryotic cells display a wide range of linker DNA length, which differs between species and among various cell types within one organism (reviewed in Ref. 2).

A single molecule of linker histone, which is often collectively called H1, is bound on the surface of the nucleosome. It interacts with DNA where it enters and exits the nucleosome, thus preventing the unpeeling of the DNA from the histone octamer.[3,4] The metazoan linker histones have a three-partite structure, consisting of a short N-terminus of 20–40 aa, a globular domain of ~60 aa and a long, flexible C-terminus of ~100 aa that constitutes almost half of the linker histone. The C-terminus is enriched in lysines, which makes it a highly

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Abbreviations used: NRL, nucleosomal repeat length; MNase, micrococcal nuclease; ssDNA, single-stranded DNA; PCA, perchloric acid.

positively charged protein.[5–7] The presence of H1 modulates the structure of the chromatin fiber.[8] Previous studies have shown that the total level of H1 affects the linker DNA length and thus also the nucleosomal repeat length (NRL). The reason is that more negative charge provided by DNA phosphate backbone is needed to neutralize the positive charge provided upon H1 binding to the chromatin.[8–10] Thus, cells with a higher H1-to-nucleosome ratio tend to have a longer NRL, and a reduction in the H1 content leads to a reduction in NRL.[2,11,12] A shorter NRL is often a characteristic of active chromatin domains and/or rapidly growing cells, such as ES cells. In contrast, mature cells with more compact chromatin tend to have longer NRL.[13,14] Here, we use the H1-induced change in the NRL as readout for H1 binding and for its effect on the chromatin structure.

Multicellular organisms have several different linker histone subtypes present simultaneously in the same cell type. There are at least eight subtypes in mice and up to eleven subtypes in humans. The sequence of the individual variants is more conserved between the species than within one species. Within one species, the H1 globular domain tends to be highly conserved while the N- and C-terminal domains are more divergent. The C-terminal tail comprises much of the heterogeneity between subtypes (reviewed in Ref. 15). While some H1 subtypes are specific for certain cells, others are present in almost all cell types. In mammals, $H1^0$ is predominantly found in terminally differentiated cells, while H1oo is specific for oocytes and the H1t and H1T2 variants are present during mouse spermatogenesis in male germ cells.[15–17] In both humans and mice, there are five somatic replication-dependent subtypes: hH1.1–hH1.5. While the expression of hH1.1 seems to be restricted to the testes, thymus and spleen, hH1.2, hH1.3, hH1.4 and hH1.5 have been found in most, if not all, somatic cell types. H1.2 and H1.4 are the predominant variants in most of the cell types that have been studied.[15,18–20]

The important questions whether this heterogeneity of the linker histone subtypes is functionally significant and, if it is, what role each subtype has in the formation and maintenance of the chromatin fiber still remain unanswered. The sequence conservation of the different subtypes between species argues for distinct functions for each subtype.[15] On the other hand, knockout studies in mice showed that the deletion of $H1^0$ alone or in combination with one of the somatic subtypes H1.2, H1.3 or H1.4 only results in compensatory elevated levels of synthesis of the remaining subtypes.[21] The deletion of H1.2, H1.3 and H1.4 yields a 50% reduction in the total H1 content compared to the wild type and embryonic lethality during mid-gestation.[21,22] Interestingly, this also leads to a significantly shorter NRL compared to the wild type. These results indicate that a correct total level of H1 is crucial for mammalian development and that the different subtypes are able to replace each other to a certain extent, that is, are functionally redundant. In contrast, other studies point toward more individual roles for different subtypes.[23,24] For example, the lack of different H1 subtypes enhances age-dependent silencing of a transgene.[25] Additionally, some H1 subtypes have been found to be associated with other regulatory proteins, which may partially explain their varying effect on gene regulation.[26,27] In line with specific functions for H1 subtypes, multiple studies reported that individual somatic linker histone subtypes localize to different chromatin domains, and it was recently shown that the pattern of covalent modifications differs between subtypes.[28–30] While previous studies revealed an overall correlation between high levels of H1 and longer NRL, the mixture of subtypes present in most cell types makes it difficult to study the role of each individual subtype.

In this study, we have utilized oocytes from the African clawed frog, *Xenopus laevis*, to address the effect of different H1 subtypes on the chromatin structure. *Xenopus* oocytes are large cells that can be used as *in vivo* test tubes to study DNA–protein interactions.[31,32] Protein(s) can be expressed in these cells via cytosolic microinjection of *in vitro* synthesized mRNA. Reporter DNA, which is provided by nuclear injection in single-stranded form, undergoes chromatin assembly during second-strand synthesis within several hours.[33] In this way, the interaction between chromatinized reporter DNA and a DNA-binding protein of interest can be studied.[34]

Xenopus oocytes lack somatic linker histones, although they do contain an oocyte-specific maternal linker histone, B4,[35,36] which, however, does not compete with H1 for chromatin binding.[37] As increased amounts of linker histone were expressed in the cell, the H1 binding was followed by the gradual increase of NRL after micrococcal nuclease (MNase) digestion and confirmed by chromatin immunoprecipitation. This increase in the NRL is saturable,[37] arguing for one specific H1-binding site per nucleosome in agreement with the *in vitro* data.[38] H1 binding is specific since NRL remained constant within a wide range of apparent H1: nucleosome ratios. However, at high concentrations, H1 starts to binds unspecifically, and the regular nucleosome ladder is then gradually replaced by a smear.[37]

Here, we compare the effect on the nucleosomal spacing of the chicken H1 variant H5 and the *X. laevis* linker histones $H1^0$ and H1A. We have also analyzed the effect of the ubiquitously expressed human somatic subtypes hH1.2, hH1.3, hH1.4 and hH1.5 and demonstrate that they can specifically regulate chromatin structure. All variants except

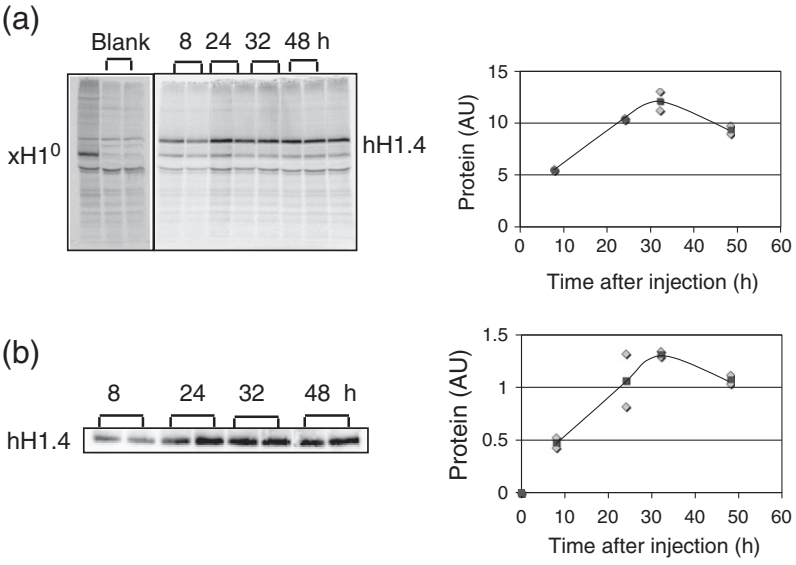

Fig. 1. The protein level reaches a maximum at 32 h after mRNA injection. (a) Oocytes were injected with 2.3 ng of mRNA coding for HA-hH1.4 and incubated in media containing [^{14}C]lysine. Oocytes were then harvested 8, 24, 32 and 48 h after injection and extracted with 5% PCA.[39,40] Homogenate from oocytes injected with 2.9 ng of xH1^{0} mRNA in 23 nl and harvested 48 h after injection was added to each sample as a loading control. (b) The intracellular H1 levels at different time points after injection were confirmed by Western blotting using anti-HA antibody (Abcam).

hH1.5 resulted in a saturable increase in NRL. Our data show that the differentiation-specific subtypes chH5 and xH1^{0} together with the human somatic subtype hH1.4 and the *Xenopus* somatic subtype H1A provide the most robust effect on NRL with an increase of 13–20 bp. Interestingly, both hH1.2 and hH1.3 result in only about half the effect as compared to hH1.4, that is, an increase of 5–7 bp in NRL. Thus, our data show for the first time that the human somatic subtypes have different effects on the chromatin structure *in vivo*, which indicates distinct roles in local chromatin structure for these subtypes.

Results

Experimental design

The linker histones were expressed in the *Xenopus* oocytes by cytosolic injection of the corresponding *in vitro* synthesized mRNAs. To monitor the intracellular level of linker histone over time, we performed a time-course study (Fig. 1). Phosphoimager analysis of the [^{14}C]lysine incorporation after SDS-PAGE showed that the H1 level in oocytes increases until 32 h after the injection and then starts to decline (Fig. 1a). To confirm that the observed decline was not due to [^{14}C]lysine deficiency in the media, we also monitored the amount of linker histone hH1.4 by Western blotting (Fig. 1b). This approach indicated essentially the same result. We concluded that the [^{14}C]lysine incorporation could be used for determining the relative level of expressed H1 proteins in our experiments. The level of expressed protein is presented in arbitrary units, and thus, the particular unit number in different experiments has no relation to absolute molar amounts or concentrations, but only to their ratios and relative amounts. Although we have standardized the exposure time of the gels, the capacity of the oocytes to synthesize protein and to incorporate ^{14}C-labeled lysine may vary from one batch of oocytes to another. Hence, the same amount of injected mRNA may result in a different protein amount in different experiments. Thus, the relative protein amount of H1 subtypes can only be compared within experiments but not between them. It should also be noted that different H1 variants have a different number of lysines. This has been accounted for when comparing the relative protein expression levels.

We routinely inject 7 ng of single-stranded DNA (ssDNA) along with [α-^{33}P]dCTP, which is incorporated into the DNA during second-strand synthesis and thus uniformly labels the DNA[33] for later detection. This results in 12–13 ng of chromatinized double-stranded DNA since the level of recovery was found to be around 90%. The *Xenopus* oocytes contain 12 pg of chromosomal DNA; thus, the reporter DNA will be the dominant one in the cell, constituting more than 99% of the nuclear DNA. Digestion of chromatin with MNase results in a characteristic cleavage pattern, that is, a nucleosomal ladder, due to the ability of MNase to cleave the linker DNA between the nucleosomes. The DNA fragments that resulted from MNase cleavage were separated on an agarose gel (Fig. 2a). The gel was dried, and NRL was analyzed using the Image Gauge Software (Fujifilm) (Fig. 2b). The expression of linker histones in the oocytes results in a reduction in electrophoretic mobility as manifested by a distinct upward shift of the MNase ladder thus reflecting the H1-induced increase in the NRL

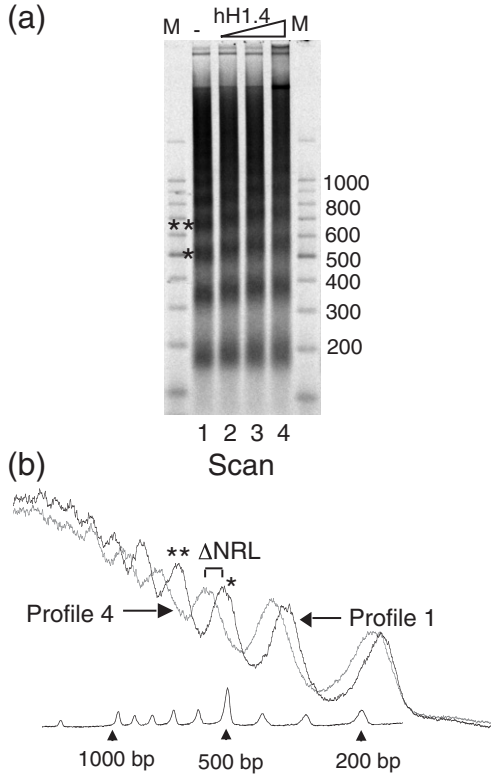

Fig. 2. MNase digestion pattern evaluated by the use of a computer-based curve-fitting method. (a) Digestion pattern of the *in vivo* assembled chromatin after the addition of increasing concentrations of hH1.4. Groups of 20–25 oocytes were injected with rising concentrations of mRNA coding for hH1.4, and after 4–6 h of incubation, 7 ng of M13 ssDNA was injected into nucleus together with [α-^{33}P]dCTP. Twenty-four hours later, the oocytes were subjected to MNase digestion, and the subsequent fragments were separated on an agarose gel. The trinucleosomal and tetranucleosomal bands are labeled with * and **, respectively. (b) Scan of the MNase digestion pattern of lanes 1 and 4 in Fig. 1a together with the DNA marker.

(compare lanes 1 and 4 in Fig. 2a and b). One issue when determining the NRL is bending or "smiling" of the gel; to avoid this problem, we used multiple DNA marker lanes, and we compared each MNase lane to the closest marker. The degree of MNase digestion can be influenced by a number of different factors, such as ionic conditions, and higher amount of MNase can cause trimming of the linker DNA. Precautions were taken to minimize these effects: we always use the same buffer, the same batch of MNase and a standardized homogenization procedure. We also used the same amount of MNase for all samples. Each group of oocytes was divided into three parts and was digested individually in order to strengthen our data by statistical evaluation of each data point.

An unbiased determination of the H1-induced effect on the MNase digestion pattern can be a challenge, since the location of the maxima of the peaks from the MNase cleavage profiles is difficult to determine with high precision. Thus, we used a computer-based curve-fitting algorithm utilized by program PeakFit® (Seasolve Software Inc., USA) in order to determine the position of each maximum and assign it a value that corresponds to its electrophoretic mobility. The NRL can be determined (Supplementary Fig. 1) by comparing the obtained values to those of the DNA standard. This process renders an unbiased analysis of the MNase-induced ladder, and the method was used throughout this study to monitor the H1 effect on the NRL. We calculated the NRL according to the mobility of the trinucleosomal band and, when possible, confirmed it by using the tetranucleosomal band (marked with * and **, respectively, in Fig. 2a). In studies by Woodcock *et al.*,[2] the NRL was calculated via plotting the DNA length of the mononucleosomes, dinucleosomes, trinucleosomes and tetranucleosomes followed by NRL prediction from the slope of the resulting line. We compared the calculations of NRL predicted from linear regression lines and found them not to differ significantly from those obtained by the method used in this paper (Supplementary Fig. 1c).

Effect of hH1.4 on the chromatin fiber

H1.4 is highly expressed in most human cells, and along with H1.2, it is the predominant H1 subtypes in humans.[18] H1.4 was shown to be essential to the survival of the human ductal breast epithelial tumor T47D cells.[24] Here, we addressed its effect on the chromatin structure by expressing it in *Xenopus* oocytes (Fig. 3). The resulting MNase-induced ladder shows a gradual increase in NRL as a function of an increasing concentration of hH1.4 in the cells (Fig. 3a). To measure the relative amount of expressed protein, we incubated seven oocytes from each pool injected with increasing amounts of hH1.4 mRNA in [^{14}C]lysine-containing media, followed by linker histone isolation by perchloric acid (PCA) extraction.[39,40] A relative amount of protein is difficult to measure at the lowest levels of expression due to a low signal-to-noise ratio at this range of protein concentrations. Thus, the amounts of histone H1 expressed after the injection of the two smallest doses of mRNA were estimated by linear extrapolation (Fig. 3b).

An analysis of the digestion pattern by using the trinucleosomal band showed that the expression of hH1.4 resulted in a total increase of NRL from 164.8 ± 2.2 bp (standard deviation, $n = 3$ for all samples) to 183.3 ± 1 bp, that is, an increase of roughly 18.5 bp (Fig. 3c, left diagram). The fact that the NRL plot reached a plateau demonstrates that

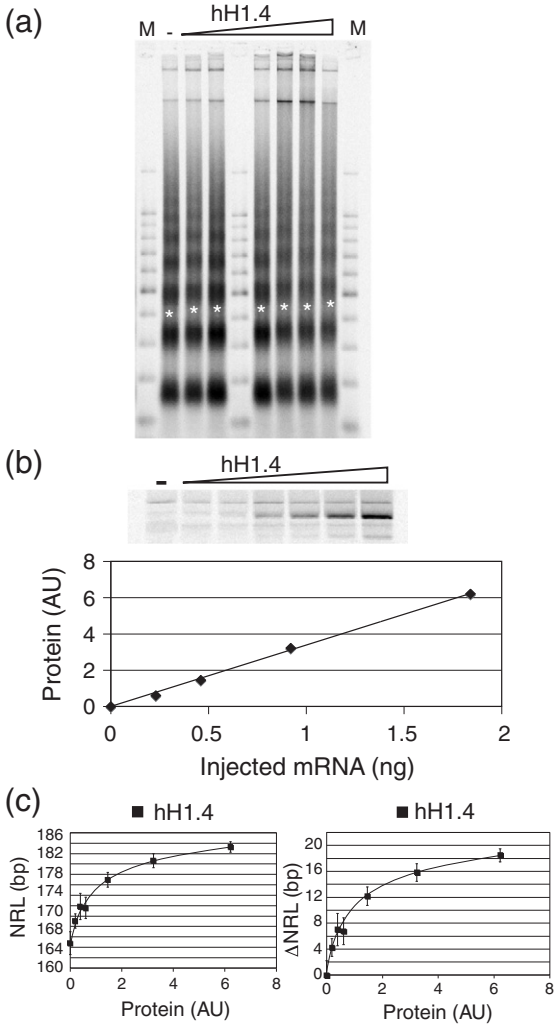

Fig. 3. A shift in NRL when hH1.4 is added is detected with MNase digestion. (a) Groups of 20–25 oocytes were injected with 1.84, 0.92, 0.46, 0.23, 0.12 and 0.06 ng of mRNA coding for hH1.4. (b) The protein level is monitored by incorporation of ^{14}C-labeled lysine, and the result is analyzed by autoradiography after SDS-PAGE. The graph shows the protein level as a function of mRNA concentration injected. Amounts of histone H1 expressed after injection of the two smallest doses of mRNA were estimated by extrapolation. (c) Addition of rising concentrations of linker histone increases the nucleosome repeat length until the DNA is saturated with hH1.4. hH1.4 gives an increase from 165 ± 2.2 bp to 183 ± 1.0 bp (left diagram), that is, an increase of roughly 18.5 ± 2.4 bp (right diagram). The standard deviation was calculated using *T*-test online (http://www.quantitativeskills.com/sisa/statistics/t-test.htm).

the hH1.4-induced effect on the NRL is saturable and indicates that the binding is specific. Such an outcome has been previously demonstrated *in vivo* and *in vitro*.[37,38] It should be noted, though, that all DNA–protein interactions also involve a nonspecific component that influences our estimation of NRL. That infers that the saturation curve will asymptotically tend to approach horizontality. We note that the NRL, in the absence of any expressed H1, may vary from one experiment to another. In most cases, it was around 160–165 bp. The final increase in NRL for a certain linker histone subtype can also vary. We attribute the observed differences to biological variations in the oocytes that were obtained from different frogs. Another contributing factor is the potential difference under experimental conditions. Due to these variations, a comparison of the effect of different linker histones on chromatin structure needs to be conducted within the same experiment. Therefore, hH1.4 has been chosen to serve as a reference in the experiments where other linker histone subtypes were studied in order to address how different subtypes behaved in comparison to hH1.4. Since we were first and foremost interested in comparing the effect on chromatin structure caused by the different linker histones, we have, in the following figures, only included the increase in NRL (ΔNRL) (Fig. 3c). The NRL in the control, where H1 was not expressed, and the NRL at saturating conditions for each linker histone type are summarized in Supplementary Tables 1 and 2.

The hH1.4 construct was originally made with 2× HA-tags that were fused to the N-terminal domain, resulting in addition of 18 aa. Since the tagged and the untagged forms showed similar increase in NRL (see below), we concluded that the HA-tag does not affect H1's ability to modulate the chromatin structure. Therefore, we decided to use the HA-tagged hH1.2, hH1.3 and hH1.5 for our studies. hH1.4, xH1A, xH1^0 and chH5 were all studied in their wild-type forms.

xH1A renders a similar increase in NRL but has lower affinity to chromatin as compared to hH1.4

Next, we studied the effect of the *X. laevis* linker histone xH1A on the NRL. *X. laevis* has six linker histone subtypes: three somatic variants (xH1A, xH1B and xH1C), one oocyte specific (B4), one sperm specific (H1fx)[41] and a replacement variant (xH1^0). Previous studies showed that the somatic variants are expressed in all *Xenopus* tissues examined so far, with H1A being the dominant one.[42] The effect of xH1A is shown in Fig. 4. xH1A results in a final increase of roughly 15 bp. In the same experiment, hH1.4 resulted in a final increase of 16.6 ± 1.4 bp. The scans of the lanes with the highest histone H1 concentration in relation to the closest size marker are shown in Supplementary Fig. 2. Thus, hH1.4 and xH1A resulted in the same increase in NRL. However, as can be seen (Fig. 4c), xH1A has a lower affinity when compared to hH1.4, since more protein is needed to reach saturation.

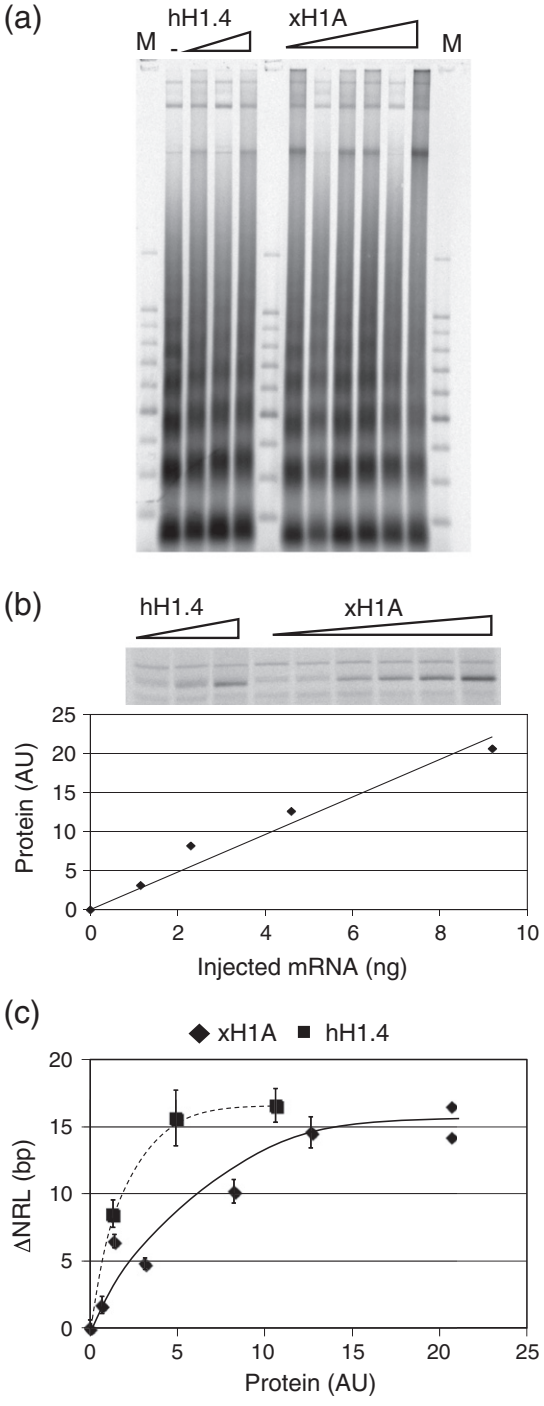

Fig. 4. *Xenopus* H1A results in the same increase compared to hH1.4. (a) Groups of 20–25 oocytes were injected with 9.2, 4.6, 2.3, 1.2, 0.6 and 0.3 ng of mRNA coding for xH1A and with 1.4, 0.7 and 0.35 ng of mRNA coding for hH1.4. (b) Expression of xH1A as monitored by incorporation of ^{14}C-labeled lysines. The two lowest protein values for xH1A were determined by extrapolation. (c) Saturation curve for xH1A. xH1A results in an increase of 15.3 ± 1.1 bp (average deviation, $n = 2$; one sample of the triplicates was removed due to gel damages), while hH1.4 results in an increase of 16.6 ± 1.4 bp.

Xenopus H1^0 and chicken H5 induce approximately the same increase in the NRL as human H1.4

While xH1A is present in most tissues, the replacement variant xH1^0 is only present in terminally differentiated cells.[42] This suggests a distinct structural role for this subtype in chromatin organization. We assessed the effect of xH1^0 on NRL and compared it to that of hH1.4. It should be noted that xH1^0 exists in two variants, xH1^0-1 and xH1^0-2.[42,43] In this study, xH1^0-2 has been studied. In line with our previous data,[37] the expression of xH1^0 yielded an increase of 12.9 ± 1.6 bp in the NRL as seen in Fig. 5. hH1.4 in the same experiment generated an increase of 11.5 ± 1.0 bp. xH1^0 and hH1.4 thus affect the NRL in roughly the same manner.

Just like xH1^0, chH5 is a differentiation-specific linker histone subtype. It is found in the very repressive chromatin of chicken erythrocytes.[44,45] In our experimental system, the expression of chH5 in *Xenopus* oocytes resulted in a total change in NRL of 21.2 ± 1.5 bp (Fig. 6c). In the same experiment, hH1.4 exhibited a total increase of 17.8 ± 1.5 bp. The NRL length estimation that was based on the tetranucleosomal band showed essentially the same result (Supplementary Fig. 3a). This result may indicate that chH5 produces a slightly longer increase in NRL in comparison to hH1.4; however, it should be stressed that our method is not sensitive enough to determine these small differences with certainty. Thus, we conclude that chH5 produces approximately the same or a slightly larger increase in NRL as hH1.4.

Human somatic linker histones have different effects on the NRL

Up to this point, we had studied linker histones that differed either in terms of which species they came from or, as in the case of xH1A and xH1^0, in terms of their expression pattern. We then addressed whether the H1 subtypes that are present in the same cell types have specific effects on the chromatin structure. In this study, we focused on the human somatic linker histones. We started by comparing hH1.2 and hH1.4, which are the predominant linker histone subtypes in most human cell types and are the only variants that have been shown to be necessary for the survival of certain tissue culture cell types.[18,24] The resulting MNase patterns indicate that hH1.2 gives a relatively small increase in NRL, namely, 6.6 ± 2 bp (Fig. 7a and c), while hH1.4 expression results in an increase of 17.2 ± 1.1 bp. The analysis of the tetranucleosomal band confirmed this result (Supplementary Fig. 3b).

We continued by exploring the effect of the two other human somatic variants, hH1.3 and hH1.5. Figure 8 shows that hH1.3 gave rise to significantly

lower increase in NRL compared to hH1.4, that is, equal to 5±1.9 bp, while hH1.4 resulted in an increase of 13.8±1.3 bp. Hence, hH1.3 seems to behave quite similar to hH1.2. We have attempted to compare hH1.3 and hH1.2 in the same experiment, but our system did not appear to be sensitive enough to detect any difference in NRL between these two subtypes (data not shown). To make sure that this significantly smaller increase was not due to the presence of the HA-tag, we compared HA-hH1.2 to wt-hH1.2 (Supplementary Fig. 4). wt-hH1.2 results in a final increase of 6.5 bp, which is comparable to the effect we see with HA-hH1.2 in the same experiment (6.4 bp), and we thus conclude that the effect of the HA-tag is dispensable.

When comparing hH1.4 and hH1.5, we discovered that, at the same level of expressed protein, hH1.4 elicits a much stronger effect on the NRL in comparison to hH1.5. Although slightly more hH1.5 than hH1.4 was expressed, the hH1.5 ΔNRL curve shows no sign of saturation (Fig. 9a). Hence, from this experiment, it appeared that hH1.5 had a considerably lower affinity for chromatin binding than hH1.4.To address whether saturation could be reached, we increased the concentration of injected hH1.5 mRNA by another twofold; however, saturation was still not attained (Fig. 9b). The only effect observed was an increased smearing of the MNase pattern, which may indicate unspecific binding or an aggregation (Fig. 9c).

Taken together, these results show that the ubiquitously expressed somatic human H1 variants can have distinct effects on chromatin structure *in vivo* and different affinities for chromatin binding. hH1.4 has the strongest effect on nucleosomal spacing, hH1.2 and hH1.3 have the weakest and hH1.5 has the lowest affinity for chromatin binding. The different subtypes are aligned in Supplementary Fig. 5, and our findings are summarized in Supplementary Tables 3 and 4.

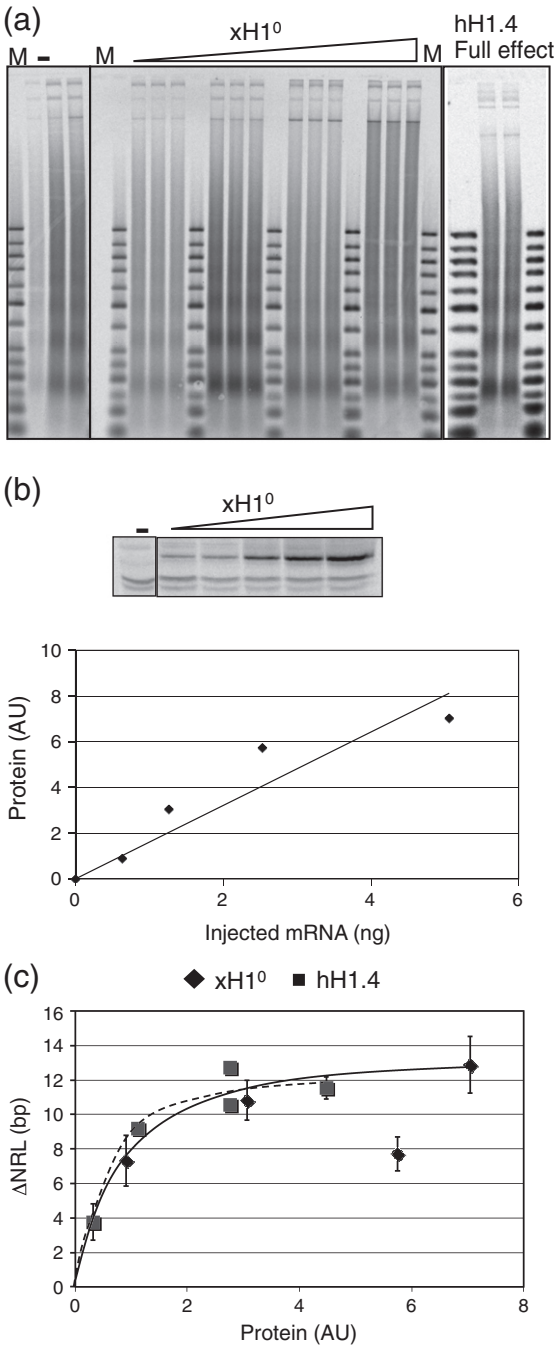

Fig. 5. *Xenopus* H1^0 results in the same increase of NRL compared to hH1.4. (a) Groups of 20–25 oocytes were injected with 5.0, 2.5, 1.27 and 0.63 ng of mRNA coding for xH1^0 and with 2.8, 1.4, 0.7 and 0.35 ng of mRNA coding for hH1.4 (gel not shown). (b) Expression of xH1^0 as monitored by incorporation of ^{14}C-labeled lysines. (c) Saturation curve for xH1^0 and hH1.4. xH1^0 results in an increase of 12.9±1.6 bp, while hH1.4 results in an increase of 11.5±1.0 bp. The NRL for the second highest protein concentration seems to be considerably shorter than expected. We occasionally found one data point in the series to be out of range both at the high and at the low concentrations of H1, this usually was due to experimental problems. As this is not seen in other experiment where xH1^0 is reconstituted in oocytes (Ref. 37 and data not shown), we have disregarded this data point and drawn the curve as it usually looks like for xH1^0.

Discussion

Here, we show for the first time in an *in vivo* system that ubiquitously expressed and redundant H1 subtypes that coexist in most cells of higher eukaryotes differ in their effects on the nucleosomal spacing *in vivo*. Our analysis indicates that the expression of most H1 subtypes, with the exception of hH1.5, resulted in a saturable increase in the NRL

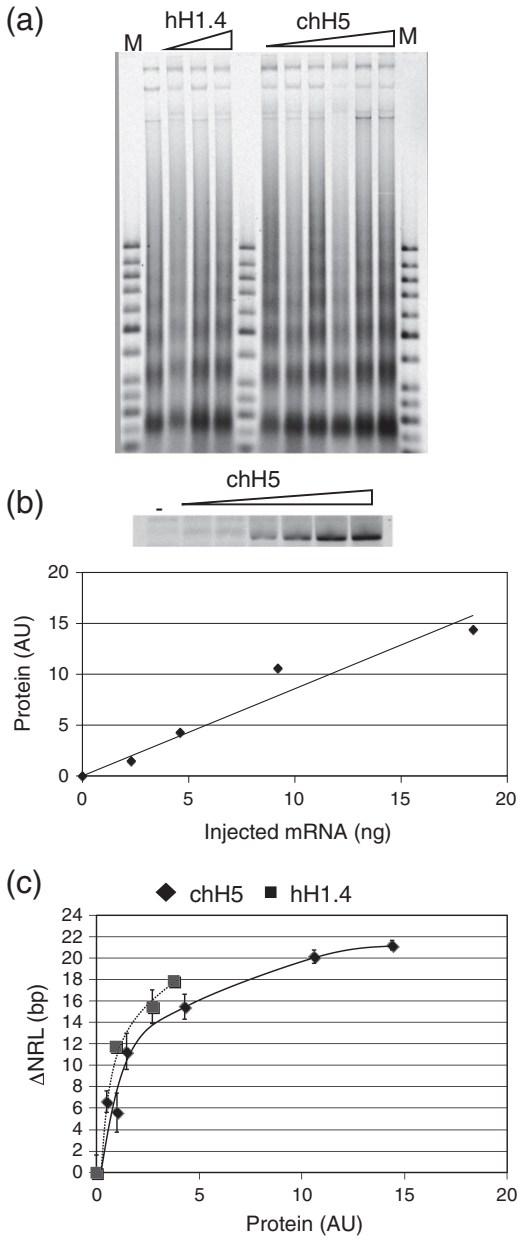

Fig. 6. chH5 results in approximately the same increase in NRL compared to hH1.4. (a) Groups of 20–25 oocytes were injected with 18.4, 9.2, 4.6, 2.3, 1.2 and 0.6 ng of mRNA coding for chH5 and with 1.4, 0.7 and 0.35 ng of mRNA coding for hH1.4. (b) Expression of chH5 as monitored by incorporation of ^{14}C-labeled lysines. The two lowest protein values for chH5 were determined by extrapolation. (c) Saturation curve for chH5 and hH1.4. chH5 results in an increase of 21.2 ± 1.5 bp, while hH1.4 results in an increase of 17.8 ± 1.5 bp.

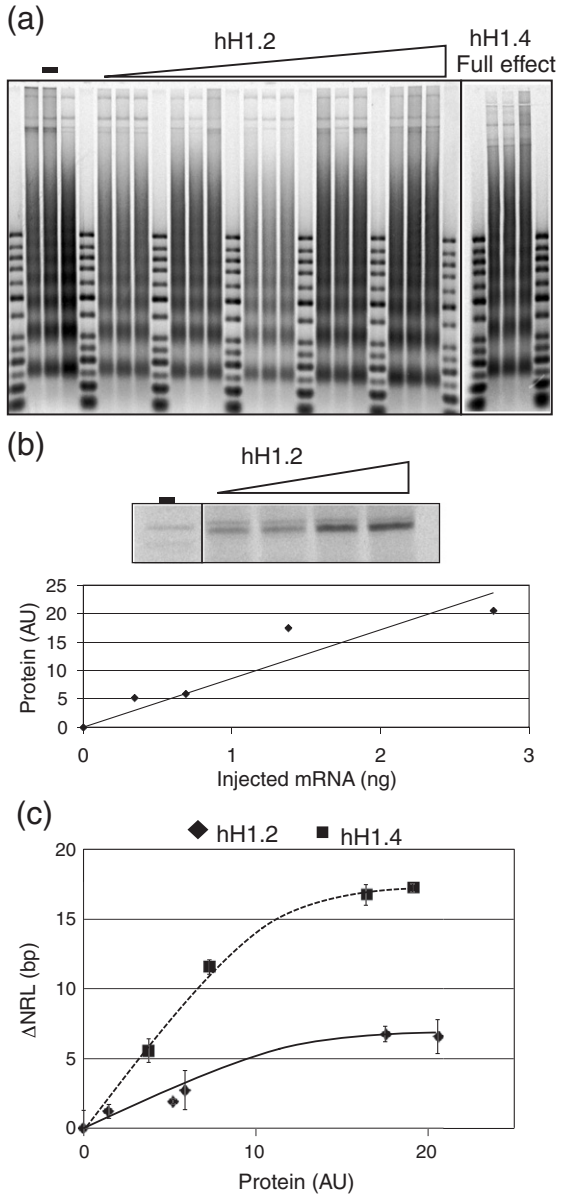

Fig. 7. hH1.2 results in significantly shorter increase compared to hH1.4. (a) Groups of 20–25 oocytes were injected with 2.8, 1.4, 0.7, 0.35 and 0.17 ng of mRNA coding for hH1.2 and with 2.8, 1.4 and 0.7 ng of mRNA coding for hH1.4. (b) Expression of hH1.2 as monitored by incorporation of ^{14}C-labeled lysines. The lowest protein value for hH1.2 was determined by extrapolation. (c) Saturation curve for hH1.2 and hH1.4. hH1.2 results in an increase of 6.6 ± 2.0 bp, while hH1.4 results in an increase of 17.2 ± 1.1 bp.

of the *in vivo* assembled chromatin. This strongly suggests that these linker histones interact with specific binding sites. hH1.4 induces an increase of approximately 13–20 bp (Fig. 3). The *Xenopus* somatic subtype xH1A had slightly lower affinity for chromatin binding but resulted in the same increase in NRL as hH1.4 (Fig. 4). The *Xenopus* differentiation-specific subtype xH1^0 had the same abilities as hH1.4, both in terms of the induced

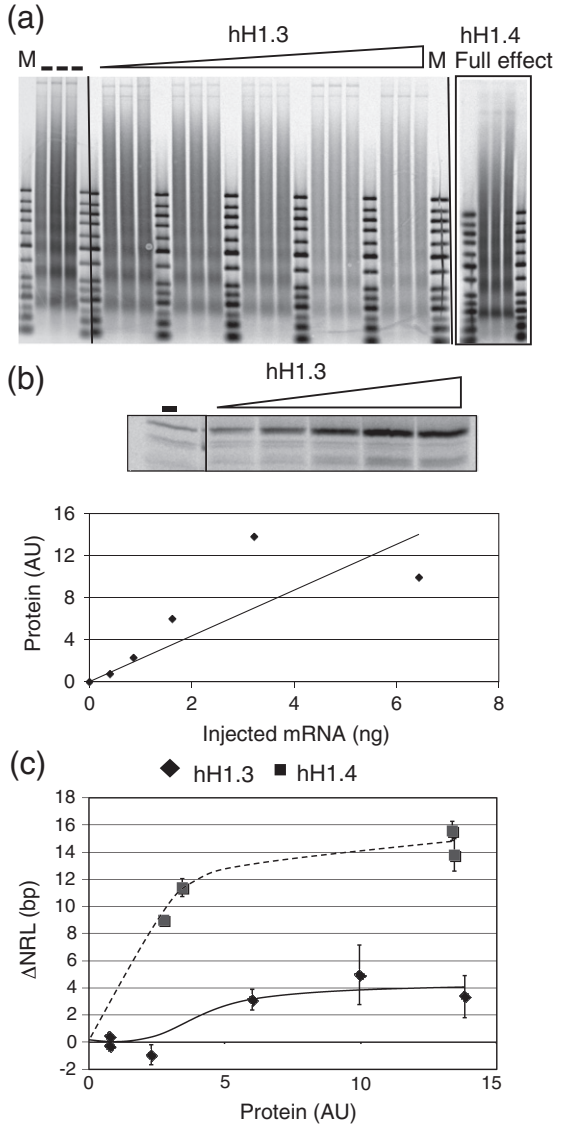

Fig. 8. hH1.3 results in significantly shorter increase compared to hH1.4. (a) Groups of 20–25 oocytes were injected with 6.4, 3.2, 1.6, 0.8 and 0.4 ng of mRNA coding for hH1.3 and with 2.8, 1.4, 0.7 and 0.35 ng of mRNA coding for hH1.4. (b) Expression of hH1.3 as monitored by incorporation of ^{14}C-labeled lysines. (c) Saturation curve for hH1.3 and hH1.4. hH1.3 results in an increase of 5 ± 1.9 bp, while hH1.4 results in an increase of 13.8 ± 1.3 bp.

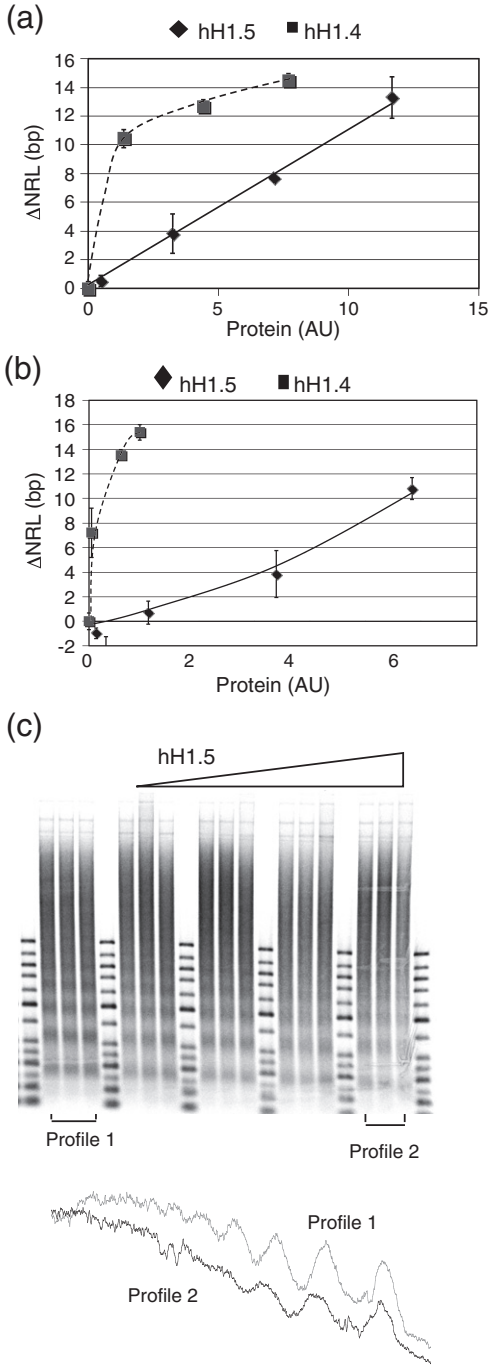

Fig. 9. hH1.5 does not give a saturating increase in NRL. (a) Groups of 20–25 oocytes were injected with 17.6, 8.8, 4.4 and 2.2 ng of mRNA coding for hH1.5 and with 2.8, 1.4 and 0.46 ng of mRNA coding for hH1.4. hH1.5 has significantly lower affinity for chromatin binding compared to hH1.4 and does not show sign of reaching saturation. (b and c) We injected 33.0, 16.0, 8.0, 4.1 and 2.1 ng of mRNA coding for hH1.5 and 4.1, 1.4 and 0.04 ng of mRNA coding for hH1.4. Still, hH1.5 does not approach saturation. (c) Digestion profiles and scans of profile 1 (control without H1 mRNA injected) and profile 2 (highest concentrations of hH1.5 mRNA injected) are shown.

increase in NRL and binding affinity (Fig. 5). The expression of chicken differentiation-specific subtype chH5 caused the same or slightly larger increase in NRL in comparison to hH1.4 (Fig. 6). Remarkably, introduction of human somatic subtypes hH1.2 and hH1.3 resulted in an NRL increase of around 4–7 bp, which is about half of that induced by hH1.4 (Figs. 7 and 8). Thus, we conclude that the biggest differences, in terms of both effect on NRL and affinity for chromatin binding, were found

between hH1.4 and other somatic human histone H1 subtypes. This observation supports the idea of a specific role in chromatin structure for different histone H1 subtypes that coexist in the same cell.

Although the *Xenopus* oocyte is a powerful system for chromatin and DNA–protein interaction studies, it has limitations. One concern regarding its suitability as a tool for H1–chromatin interaction studies is the differences between *Xenopus* core histones and human core histones. It should be stressed that the *Xenopus* core histones have a high degree of structural similarity to the human core histones, differing in only a few amino acids. However, the oocytes have also been found to contain a large amount of H2A.X.[41,46] This histone subtype has been proposed to act as a signaling molecule and may be involved in the modulation of cellular response to high rate of cell division during early stages of development. While one cannot exclude the possibility that this may influence our results, it is highly unlikely that the chromatin assembled after DNA injection has any adverse effects on H1 binding or poses any hindrance to H1 studies. Our results that demonstrate saturable, and hence specific, binding of most of the here tested linker histone subtypes to the *in vivo* assembled chromatin serves as a proof of concept and argues for its usefulness for such studies.

NRL in the cell is determined not only by the presence of linker histones but also by other factors that can modulate the distance between nucleosomes such as precise ionic conditions, the protein modification status (e.g., phosphorylation of histone tails) and the presence of other proteins.[47] Ubiquitous nuclear proteins such as MeCP2[48] or HMG (*h*igh *m*obility *g*roup) proteins are known to interact with H1 and/or compete with it for binding sites.[49–51] Interestingly, in *Xenopus* oocytes, the NRL in the absence of exogenously expressed H1 was found to be 160–165 bp, as in *Saccharomyces cerevisiae* devoid of canonical linker histones. This is in agreement with our previous finding that the *Xenopus* oocyte-specific linker histone B4 is not involved in the formation of nucleosomal structure, as it does not compete for binding with exogenously expressed histone xH1^0.[37] Hence, it seems unlikely that other proteins may affect the NRL in the absence of histone H1. *Xenopus* oocytes do, however, contain HMG proteins,[52] and it cannot be excluded that these proteins compete with exogenous H1 for binding. This is why histone hH1.4 was included as a reference in all our experiments, and thus, all H1 subtypes were tested against the same background of potentially competing proteins. It should also be mentioned that the *Xenopus* oocyte contains only 12 pg of chromosomal DNA. We routinely inject 7 ng of ssDNA. Thus, the injected chromatinized DNA will be dominant, constituting 99.9% of the DNA content in the cell. Under these conditions, the ratio of potential competitors to nucleosome will be dramatically reduced, and thus, chances of these proteins to compete with histone H1 would be very low. We conclude that the presence of potential competitors cannot change our conclusions regarding the effect of different histone H1 subtypes on NRL. However, we cannot formally exclude but consider it to be unlikely that observed differences in affinity for chromatin binding (Figs. 4 and 9) are influenced by differences in the ability of various linker histone subtypes to compete with other proteins, rather than being solely due to intrinsic binding affinity. This, however, cannot affect our estimations of NRLs since those were determined at saturating conditions.

What do different effects on NRL tell about the potential function of each linker histone subtype? The chicken erythrocyte is a terminally differentiated cell, with very compact and inert chromatin with a long NRL and a total of 1.3 molecules of linker histones per nucleosome, that is, 0.4 chH1 and 0.9 chH5.[44,53] During erythropoesis, the NRL increases from 190 bp to 212 bp. This increase is correlated with the rising concentration of H5, but not H1, in these cells.[45] During the maturation of *Xenopus* erythrocytes, the content of xH1^0, namely, xH1^0-2 that was used here, increased threefold. It has been shown to be the only H1^0 subtype present in these cells.[54,55] This argues that the function of xH1^0 in *Xenopus* erythrocytes may be analogous to that of chH5 in chicken erythrocytes. In line with their roles in the formation of tightly packed chromatin, xH1^0 and chH5 were found among the subtypes that produced the most robust increase in NRL (Figs. 5 and 6). Interestingly, the somatic subtypes *Xenopus* xH1A and human hH1.4 had a similar effect on NRL as xH1^0 (Figs. 4 and 5). xH1A is present in virtually all cell types from the embryo stage and onwards in the frog,[42] and hH1.4 is present in all cell types studied in humans.[15,18] However, xH1A is also known to have a role in the silencing of chromatin. xH1A starts to accumulate in the embryo during early gastrula, and at this stage, it has a crucial role in the silencing of oocyte-type 5S RNA synthesis.[56–58]

The similarities between hH1.4 and the histone subtypes with known heterochromatic and gene-silencing behavior such as chH5, xH1^0 and xH1A could indicate a role in the formation/maintenance of heterochromatic regions for hH1.4. Both hH1.2 and hH1.3 result in about half of the increase in NRL in comparison to hH1.4 (Figs. 7 and 8). Such evidence points toward a potential role for hH1.2 and hH1.3 in the formation of less dense chromatin structures or domains. This would agree with a study of green fluorescent protein-tagged H1 in mouse fibroblasts that indicated H1.4 to be localized to heterochromatic regions, while H1.2 and H1.3 were enriched in euchromatin.[59] An *in vitro* study also classified hH1.4 (as well as hH1^0) as a strong chromatin condenser, hH1.3 as intermediate and

hH1.2 (and hH1.1) as weak chromatin condensers.[60] The overexpression of mouse $H1^0$ and mouse H1.2 in cultured mouse fibroblasts showed that, while both variants resulted in an increase in NRL, the $H1^0$ overexpression resulted in significantly more dense chromatin resistant to MNase digestion.[61] One can assume that if some subtypes have a primary role in the formation and/or maintenance of dense chromatin, then these subtypes also would be more repressive in terms of transcription. Indeed, chH5 was shown to be more effective as an inhibitor of transcription when introduced in proliferating L6 rat myoblasts compared to chH1a and chH1b.[62] Knockdown of H1 subtypes one by one in human breast cancer cell lines points toward a more activating role for hH1.2 in comparison to hH1.4.[24] A study using the mouse ES knockdown cells revealed that the depletion of H1.4 but not H1.2 significantly attenuated the age-related silencing of the human β-globin transgene, confirming the more repressive role of H1.4 compared to H1.2.[25] Despite obvious similarities to H1.2, H1.3 knockdown was shown to have the same effect on gene expression as a lack of H1.4. Chromatin immunoprecipitation experiments at selected regions indicated a lack of H1.4 and H1.3 in active chromatin and chromatin poised for transcription.[63] These results regarding hH1.3 may point toward a more intermediate role for this subtype.

In our experimental setup, the mode of interaction of hH1.5 with chromatin was found to be quite different from that of hH1.4, and other linker histones studied as hH1.5 had a significantly lower affinity to chromatin and did not result in a saturable increase in the NRL (Fig. 9). Thus, our results differ from the data obtained by the use of fluorescence recovery after photobleaching, where hH1.4 and hH1.5 were shown to have almost identical recovery curves.[59] Other studies also point toward similarities between hH1.4 and hH1.5, and both are enriched in heterochromatin.[59] A study using *Drosophila* embryo extract also showed similar binding curves for hH1.4 and hH1.5, and they were both characterized as strong condensers of chromatin *in vitro*.[59,60] Our data, however, indicate that there are differences between hH1.4 and the other human H1 subtypes on one hand and hH1.5 on the other when it comes to interaction with chromatin. The apparent differences in binding affinity could of course be attributed to difference in the ability to compete with other proteins for binding sites, as discussed above. *In vitro* data have shown that, in the absence of the histone chaperone xNAP-1, the *Xenopus* egg-specific linker histone B4 binds nonspecifically to chromatin. This is seen as smearing in the MNase pattern[64] and resembles the pattern that we observed for hH1.5 in Fig. 9c. Thus, one possible explanation for this discrepancy in binding abilities between hH1.5 and other histones is that H1.5 needs some kind of chaperone, cofactors or specific covalent modifications to support proper binding to chromatin that are missing in *Xenopus* oocytes. Data by Talasz *et al.* showed a significantly reduced affinity of H1.5 for binding to reconstituted nucleosomes and naked DNA,[65] and Talasz *et al.* and Orrego *et al.* reported that hH1.5 has an intermediate affinity for long chromatin fragments at physiological salt concentrations.[65,66] Thus, *in vitro* studies show a lower affinity of hH1.5 for chromatin binding in comparison to studies performed in cells or a cell-like environment, giving further support to the concept that some additional factor is required for its incorporation into chromatin.

We do not know if H1 is posttranslationally modified in the oocytes. Thus, we cannot exclude that the properties of H1 are influenced by posttranslational modifications that are absent in our system. However, it is of interest to note that the modification pattern differs between hH1.2 and hH1.4. While both are methylated by the methyltransferase G9a, hH1.4 is methylated on K26, and hH1.2 is methylated on K187.[29] Interestingly, the heterochromatin protein-1 binds specifically to methylated K26 of H1.4, but not to methylated K187 of hH1.2. This further supports the notion that hH1.4 and hH1.2 are acting in different types of chromatin.

Earlier studies have shown that some linker histone variants bind to naked DNA with a higher degree of cooperativity compared to the others. This was shown, for example, for chH5 compared to chH1.[67] However, whether the linker histone also binds cooperatively to chromatin remains to be investigated.[68] Our results do not address this question; however, we speculate that the binding of one molecule of a particular H1 subtype may facilitate the binding of yet another molecule of the same subtype and, thus, may contribute to the formation of a local chromatin structure.

How can the differences in the ability of individual H1 subtypes to regulate nucleosomal spacing be explained? When comparing H1 proteins, their globular domains tend to be very similar while the tails, particularly the C-terminal domain, are different in length and number of positively charged amino acids. Another point of difference is the distribution of positively charged amino acids, lysines and arginines, that is, factors that have all been implicated to play a role in H1 chromatin binding affinity.[59] We found the biggest differences in terms of length and amino acid content between $xH1^0$ and chH5 on one hand and the human somatic subtypes on the other when comparing the C-terminal domains and the whole protein (Supplementary Tables 5 and 6). The same was true when comparing the distribution of positive charge and phosphorylation sites in the C-terminal domain (Supplementary Figs. 6 and 7) and when comparing

the N-terminal domains (Supplementary Fig. 8) whose function is still enigmatic. We did not observe any striking differences that could explain the different effect on the chromatin structure of the human subtypes. Hence, the amino acid composition of the H1 protein or the N- and C-termini is not the only determinants for H1–DNA interactions. Other factors that may affect this are spatial organization of the charged amino acids and the DNA-binding sites in the globular domain that have recently been shown to differ between subtypes.[69–71] A recent study has made it clear that the globular domain and the C-terminal domain of H1 bind to chromatin with an intramolecular cooperativity.

One reason for having several H1 subtypes, aside from them having different roles in chromatin structure, could be that they have different functions during mitosis and/or as signaling molecules. A study of H1 behavior during the cell cycle showed differences between subtypes.[72] H1.2, which had the smallest effect on NRL in our study, has also been shown to be involved in apoptosis signaling.[73] We speculate that weak H1.2 association with chromatin facilitates its rapid dissociation, thus making it suitable for functioning as a signaling molecule, for example, for DNA damage.

In this study, we have shown that somatic linker histone subtypes that usually coexist in the same cell type contribute differently to chromatin structure. We suggest that hH1.4, which results in an increase in NRL comparable to those of subtypes found in repressed chromatin in specialized cells, could contribute to the formation and/or maintenance of heterochromatin. Both hH1.2 and hH1.3 result in about half the effect on NRL as compared to hH1.4, and in agreement with data from others, we propose a more euchromatic role for hH1.2 and an intermediate role for hH1.3. To gain further insight into the different roles of H1 subtypes, it would be of interest to study their location(s) on a genome-wide scale.

Materials and Methods

mRNA synthesis

Constructions of the plasmids for *in vitro* production of the linker histone mRNA were generated by PCR amplification of respective cDNA clones using specific primers that generated BamHI and NotI sites in the 5′ and 3′ end of the inserts, respectively (available upon request). The resulting fragments were inserted into the RN3P vector[74,75] between the BamHI and NotI sites. A 2× HA-tag was added to the N-terminus of the human linker histones, that is, hH1.2 (GenBank: X57129.1), hH1.3 (GenBank: M60747.1), hH1.4 (GenBank: M60748.1) and hH1.5 (GenBank: X83509.1). The 2× HA-tag was subsequently removed from hH1.4. Chicken H5 (NCBI reference sequence number NP_001038138.1) is a gift from V. Ramakrishnan, and xH1A (GenBank: DQ466082) is a gift from K. Ura. *Xenopus* $H1^0$-2 (GenBank: Z71503.1) was ordered from GeneScript, USA. Each construct was confirmed by DNA sequencing, they were linearized with Asp718 and the linker histone mRNA was synthesized using mMessage mMachine® kit (Ambion #AM1348).

Xenopus oocyte preparation

The handling of the frogs and the experiments have been approved of by the Central Commission for Animal Research (Centrala Försökdjursnämnden), reference number N61/09. Preparation of *Xenopus* oocytes and oocyte injections was performed as described earlier.[76,77] The linker histone variants were expressed in the oocytes by cytosolic injection of 23 nl of mRNA solution. After 4–6 h of incubation at 19 °C, 7 ng of M13mp18 ssDNA (USB, P/N 71706) was co-injected with [α-^{33}P]dCTP (NEG613H; PerkinElmer) in a volume of 18.3 nl in the oocyte nucleus.

MNase digestion

The MNase digestion was performed ~24 h after DNA injection. At least 15 oocytes were homogenized with a Dounce homogenizer in the volume of 18 µl times the number of oocytes of 15 mM Tris–HCl (pH 8.1), 50 mM KCl, 1 mM dithiothreitol, 5% glycerol and 1 mM $CaCl_2$. Three aliquots of homogenate containing 3.3 oocytes each were digested with 5.6 U MNase (USB, P/N 70196Y) for 5 min in 15 °C. The reaction was stopped by the addition of 2× STOP solution: 10 mM Tris–HCl (pH 8.0), 10 mM ethylenediaminetetraacetic acid, 1% SDS and 0.1 mg/ml proteinase K. The samples were incubated overnight in 37 °C. The DNA was extracted using phenol:chloroform extraction and ethanol precipitation and then treated with 5 µg RNase A for at least 1 h at 37 °C. The samples were dissolved in 13 µl of 5 mM Tris (pH 8.0) and 1 mM ethylenediaminetetraacetic acid, and 5 µl of 6× Orange DNA loading dye (Fermentas, #R0631) and 6 µl were run on a 1.3% agarose gel [0.5× Tris base, boric acid, EDTA (TBE)-buffer].

Analysis of NRL

The agarose gels were dried and scanned using a Fuji Bio-Imaging FLA-7000 analyzer and the Image Gauge V4.1 Software. The profiles of the MNase digestion pattern were exported as an Excel file and reconstituted in PeakFit® v.4.12 (Seasolve Software Inc., USA). The MNase digestion pattern was smoothened using the Loess algorithm (Seasolve Software Inc., USA), and the size marker ladders were smoothened using the FFT filtering (Seasolve Software Inc., USA), which is more suitable for these sharp peaks. The program assigned a value to each maximum, and the known sizes of the DNA standard were plotted as a function of these values. The equation for the graph was used to calculate NRL for the MNase digestion pattern. The standard deviation for the ΔNRL were calculated†.

†using http://www.quantitativeskills.com/sisa/statistics/t-test.htm

Quantification of expressed protein

Seven oocytes/group were incubated in oocyte media containing 3 μCi/ml of [^{14}C]lysine. The linker histones were extracted in 5% PCA, and after SDS-PAGE, the relative intensity of the band corresponding to H1 was determined in the Image Gauge V4.1 Software. Relative protein amount was determined in arbitrary units and thus cannot be directly compared between experiments. The difference in lysine content between the histones was accounted for. For the Western blot analysis, anti-HA antibody from Abcam, number ab9110, was used as primary antibody and anti-rabbit IgG from GE Healthcare, number NA934V, was used as secondary antibody. The blots were developed using the computer program IR-Las 1000 Pro ver. 2.11 (Fujifilm), and the bands were quantified using the Image Gauge V4.1 Software.

Acknowledgements

This work was supported by the Swedish Cancer Foundation (project number 110702) and the Swedish Research Council Medicine (project number K2008-66X-15337-04-3). C.Ö. was supported by Karolinska Institute funding for PhD students and the Royal Swedish Academy of Sciences.

Author Contributions. C.Ö. performed the experiments, analyzed the data and drafted the manuscript. A.I. and R.S. designed and made the H1 constructs for mRNA expression and helped discussing the conclusions. Ö.W. helped planning the work, designing the experiments and took part in writing the article. S.B. planned the work, designed the experiments, performed parts of some experiments and took part in writing the article. All authors read and approved the final manuscript.

Competing Interest. The authors declare that they have no competing interests.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2012.03.007

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