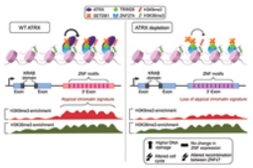
epigenetics

Taylor & Francis
Taylor & Francis Group

Epigenetics



ISSN: 1559-2294 (Print) 1559-2308 (Online) Journal homepage: http://www.tandfonline.com/loi/kepi20

The characterization of macroH2A beyond vertebrates supports an ancestral origin and conserved role for histone variants in chromatin

Ciro Rivera-Casas, Rodrigo Gonzalez-Romero, Manjinder S. Cheema, Juan Ausió & José M. Eirín-López

To cite this article: Ciro Rivera-Casas, Rodrigo Gonzalez-Romero, Manjinder S. Cheema, Juan Ausió & José M. Eirín-López (2016) The characterization of macroH2A beyond vertebrates supports an ancestral origin and conserved role for histone variants in chromatin, Epigenetics, 11:6, 415-425, DOI: 10.1080/15592294.2016.1172161

To link to this article: http://dx.doi.org/10.1080/15592294.2016.1172161

EPIGENETICS
2016, VOL. 11, NO. 6, 415–425
http://dx.doi.org/10.1080/15592294.2016.1172161

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

The characterization of macroH2A beyond vertebrates supports an ancestral origin and conserved role for histone variants in chromatin

Ciro Rivera-Casas[a], Rodrigo Gonzalez-Romero[a], Manjinder S. Cheema[b], Juan Ausió[b,*], and José M. Eirín-López[a,*]

[a]Department of Biological Sciences, Chromatin Structure and Evolution (Chromevol) Group, Florida International University, North Miami, FL, USA; [b]Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada

ABSTRACT

Histone variants play a critical role in chromatin structure and epigenetic regulation. These "deviant" proteins have been historically considered as the evolutionary descendants of ancestral canonical histones, helping specialize the nucleosome structure during eukaryotic evolution. Such view is now challenged by 2 major observations: first, canonical histones present extremely unique features not shared with any other genes; second, histone variants are widespread across many eukaryotic groups. The present work further supports the ancestral nature of histone variants by providing the first *in vivo* characterization of a functional macroH2A histone (a variant long defined as a specific refinement of vertebrate chromatin) in a non-vertebrate organism (the mussel *Mytilus*) revealing its recruitment into heterochromatic fractions of actively proliferating tissues. Combined with *in silico* analyses of genomic data, these results provide evidence for the widespread presence of macroH2A in metazoan animals, as well as in the holozoan *Capsaspora*, supporting an evolutionary origin for this histone variant lineage before the radiation of Filozoans (including Filasterea, Choanoflagellata and Metazoa). Overall, the results presented in this work help configure a new evolutionary scenario in which histone variants, rather than modern "deviants" of canonical histones, would constitute ancient components of eukaryotic chromatin.

ARTICLE HISTORY
Received 4 March 2016
Revised 22 March 2016
Accepted 24 March 2016

KEYWORDS
Chromatin; epigenetics; evolution; function; histone variants; In Vivo; metazoans; nucleosome; structure

Introduction

In eukaryotesand in some archaebacteria the DNA is associated with histones and non-histone chromosomal proteins in a nucleoprotein complex known as chromatin, which organizes and regulates the expression of the hereditary material within the cell nucleus. Among histones, the H2A family exhibits a large number of variants, and their incorporation into nucleosomes plays a critical role in chromatin dynamics.[1,2] The variant macroH2A stands out within this family as the most atypical histone known so far, consisting of a N-terminal H2A domain and a C-terminal non-histone domain (NHD) portion corresponding to the macro domain.[3] Overall, its size is approximately 3 times that of a canonical histone H2A. MacroH2A was first discovered more than 2 decades ago in rat liver nucleosomes,[3] with early cytological studies showing macroH2A enrichment at inactive X chromosomes (Xi) from female mammals.[4,5] Subsequent reports have associated this variant with different heterochromatic regions of both sexual and autosomal chromosomes in vertebrates,[6-10] revealing a significant depletion of macroH2A content in transcribed regions of many active genes.[10,11] Based on these observations, it was initially suggested that the major function of macroH2A was that of transcriptional repression. However, evidence has accumulated ever since indicating that macroH2A is associated to the active state of a subset of genes,[10,12,13] suggesting that it might play a general structural role that, if perturbed, could affect transcription.

MacroH2A displays several isoforms (Fig. 1A). Two of them, macroH2A.1.1 and macroH2A.1.2, result from alternative splicing of the macroH2A.1 gene,[14] while macroH2A.2 is encoded by an independent gene.[5] Based on their different expression patterns during vertebrate development and their distribution across tissues, it is likely that these variants are involved in specialized functions.[5,14,15] Accordingly, it has been demonstrated that macroH2As can exert large positive or negative effects on gene expression, with macroH2A.1 and macroH2A.2 acting synergistically on the expression of some genes and apparently having opposing effects on others.[16,17] The role of macroH2A in chromatin has been functionally ascribed to processes including cellular differentiation,[15,18-20] embryo development[21] and lipid metabolism.[11,17,22] Additionally, deregulation in macroH2A.1 alternative splicing has been related to the metastatic transition in several types of cancer.[23-25] Several *in vivo* and *in vitro* studies indicate that macroH2A increases nucleosome stability due to the specific structural features of its H2A domain.[26,27] It has also been shown that the structural changes resulting from the incorporation of macroH2A into nucleosomes prevent the access to chromatin by some remodeling complexes (e.g., SWI/SNF).[28] In

CONTACT Juan Ausió jausio@uvic.ca; José M. Eirín-López jeirinlo@fiu.edu
Supplemental material for this article can be accessed on the publisher's website.
*These authors are equal contributors to this work.

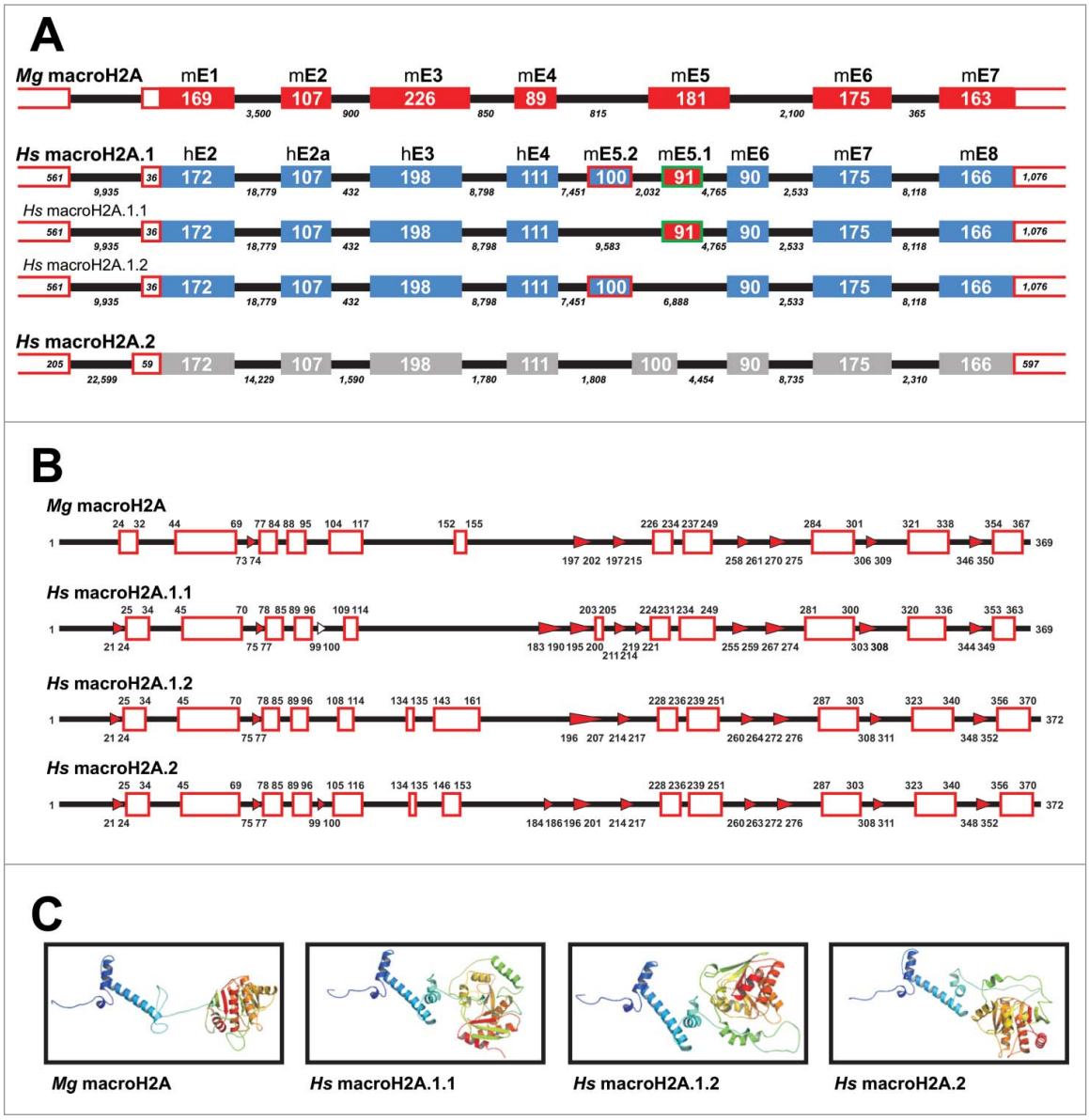


Figure 1. Gene organization and protein structure of the mussel macroH2A. A) Gene organization of human (*Hs*) and mussel (*Mg*) macroH2A genes (represented at an arbitrary scale for comparison purposes). *Hs* macroH2A.1.1 and *Hs* macroH2A.1.2 are splicing variants from the *Hs* macroH2A.1 gene. The length of exons and introns (number of nucleotides) is indicated at the corresponding positions (mE, mussel exon; hE, human exon). Exon numbering in humans was assigned after.[6] Red open boxes at 5′ and 3′ positions represent untranslated regions (UTRs), indicating their length in nucleotides. B) Secondary structure prediction for different macroH2A variants from metazoan animals including vertebrates and invertebrates. Red boxes and red arrows indicate the presence of α-helices and β-sheets, respectively, at the amino acid positions indicated. C) Predicted tertiary structure for *Mg* macroH2A [modeled using Phyre2 [73]] compared with those of human macroH2A proteins.

addition, *in vitro* studies have demonstrated the ability of the linker domain of this histone to enhance chromatin condensation in a way that resembles histone H1 and is modulated by the macro domain.[29,30] Interestingly, macroH2A is found in regions of chromatin that are depleted of histone H1.[26] Finally, its NHD has been shown to interact with transcription factors and complexes involved in the establishment of posttranslational modifications.[12,28, 31,32]

For a long time macroH2A was thought to be an invention of vertebrates, culminating (together with H2A.B) the functional diversification of variants within the H2A family.[33-36] The hypothetical existence of a functional invertebrate macroH2A bears 2 critical implications: first, the evolutionary origin of this variant would have to be redefined; second, the role of macroH2A in chromatin structure and epigenetic regulation would require further examination in a broader evolutionary context. Unfortunately, no conclusive experimental information is currently available for the non-vertebrate counterpart of this histone variant. The present work fills this gap by providing the first *in vivo* characterization of macroH2A in non-vertebrate animals. In doing so, our results shed light on the origin of this variant and its functional role in chromatin, unveiling a new evolutionary scenario in which variants, far from being "deviants," would constitute ancient components of eukaryotic chromatin.

Results

Identification and sequence characterization of mussel macroH2A gene

The complete macroH2A gene sequence obtained from the mussel *Mytilus galloprovincialis* (*Mg*) (mRNA deposited in

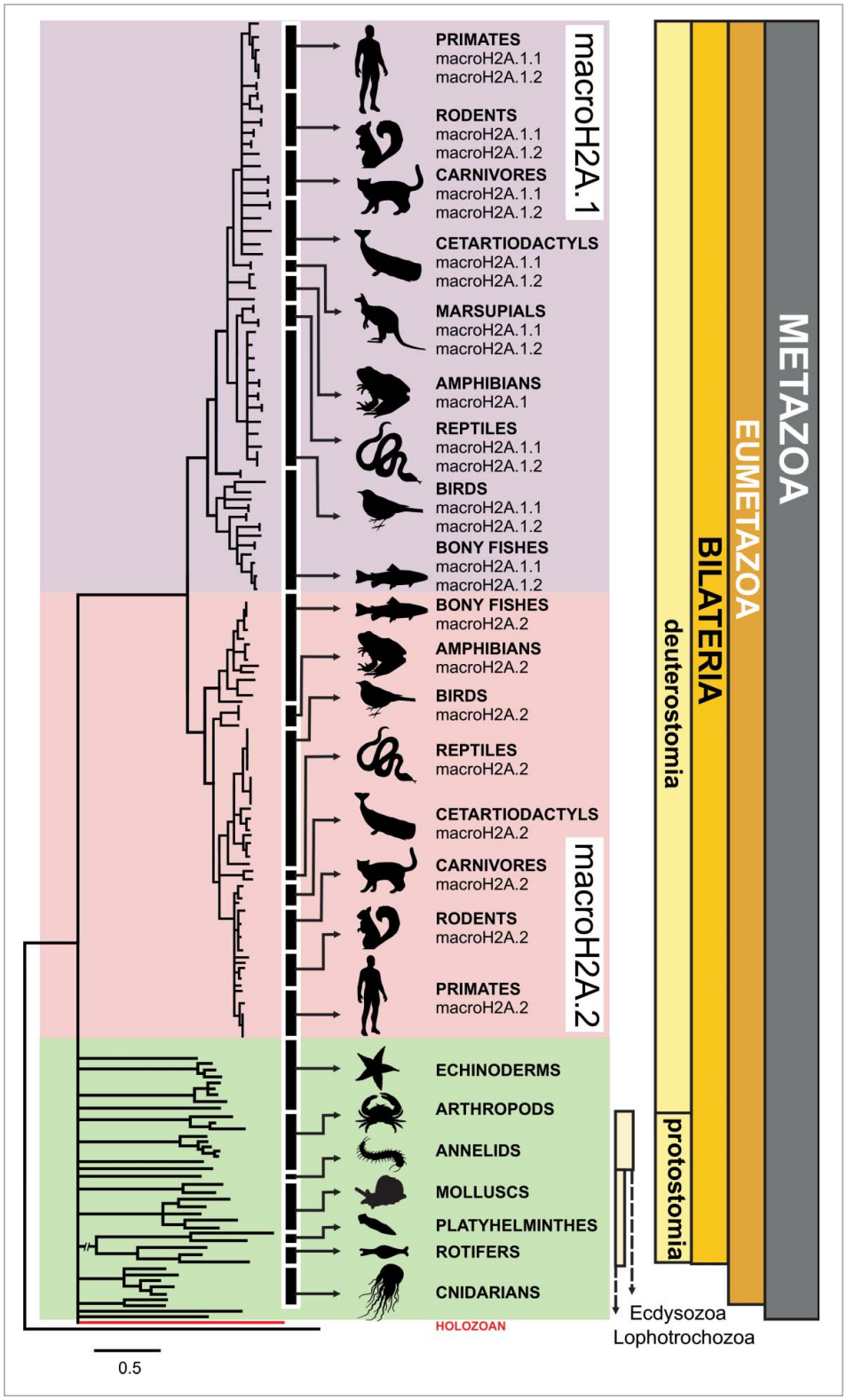


Figure 2. New evolutionary context for macroH2A. Schematic macroH2A protein phylogeny (see complete tree in Fig. S3) illustrating the wide distribution of this histone variant across metazoans and in the holozoan *Capsaspora*. The progressive specialization of macroH2A is evident in vertebrates, resulting in the differentiation of macroH2A.1 (purple) and macroH2A.2 (pink), different from macroH2A from non-vertebrates (green). The taxonomic classification of the organisms represented in the tree is indicated in the right margin of the figure. The tree was rooted with the human histone H2A.Z, as it constitutes a sister monophyletic group of macroH2A within the H2A phylogeny.[34,38]

GenBank with accession KT894822) encompasses approximately 9,600 nucleotides and its organization consist of 7 coding exons (Fig. 1A). The coding sequence of *Mg* macroH2A consists of 1,110 nucleotides encoding a 369 amino acid protein (Fig. S1). The similarity of *Mg* macroH2A with its vertebrate counterpart is further mirrored by the secondary and tertiary structures predicted based on its amino acid sequence (Fig. 1B, 1C). Like in the case of *Homo sapiens* (*Hs*) macroH2A, *Mg*

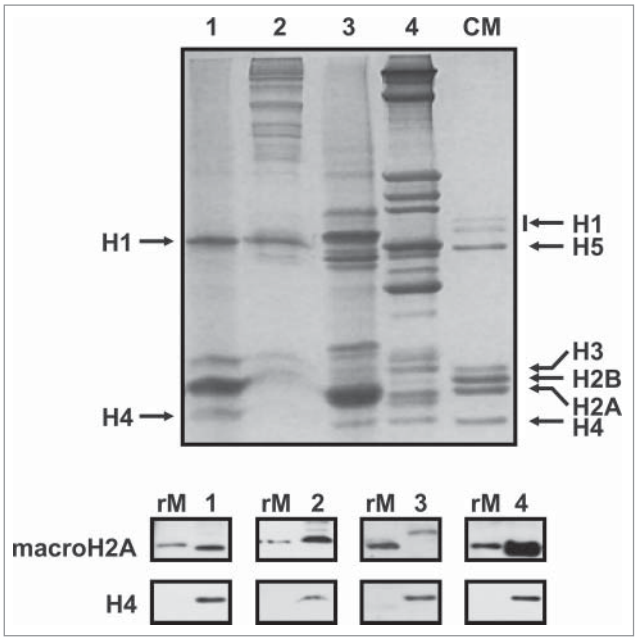


Figure 3. Detection of the macroH2A protein detection in non-vertebrates. Upper gel, SDS-PAGE of HCl-extracted histones from representative organisms including: mussel (*M. californianus*) hepatopancreas (lane 1), tick (*A. maculatum*) salivary glands (lane 2), sea urchin (*S. purpuratus*) male gonad (lane 3) and amphioxus (*B. floridae*, lane 4). CM, chicken erythrocyte histones used as marker. Lower gel, western blot analysis of the HCl-extracted proteins using the invertebrate-specific anti-macroH2A antibody (M12) developed in the present work. Mussel recombinant macroH2A (rM) was used as positive control, and anti-H4 antibody for sample normalization.

macroH2A encompasses an H2A domain (amino acids 1 to 120) displaying 58% identity with the homologous region in the canonical *Mg* H2A, followed by a basic linker region (amino acids 121 to 178) connecting the H2A domain with the macro domain (amino acids 179 to 369) (Fig. S1). As expected, the H2A domain from mussel macroH2A is more identical to its homologous region in *Hs* macroH2A.1 (75%) and *Hs* macroH2A.2 (72%) than in the canonical *Mg* H2A. In the case of the macro domain, *Mg* macroH2A shares 61% identity with *Hs* macroH2A.1.1, 55% with *Hs* macroH2A.1.2, and a 50% with *Hs* macroH2A.2. Lastly, the linker domain constitutes the most divergent region between *Mg* macroH2A and *Hs* macroH2A (Fig. S1, 17% identity with *Hs* macroH2A.1, 8% with *Hs* macroH2A.2). It appears that the identity of this linker region is determined by a variable amino acid sequence with intrinsically disordered organization and a compositional enrichment in A, K, P amino acids (see Table S1) that are reminiscent of the C-terminal tails of H1 histones.[29,30,37] Despite the low levels of similarity of these linker regions, mussel and human macroH2As all retain these characteristic structural features and hence they are most likely functionally related.

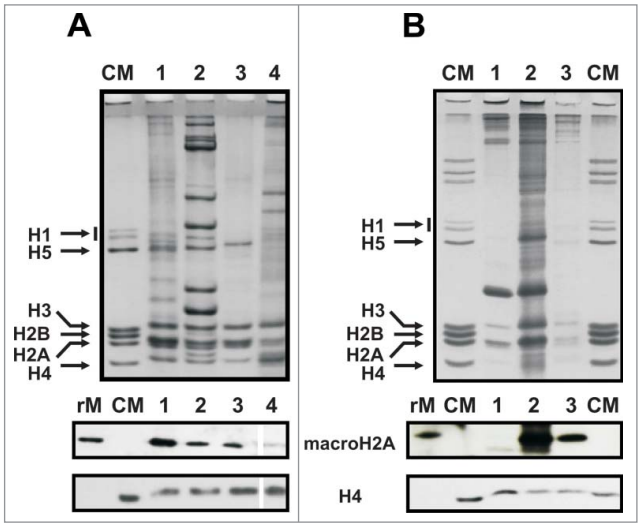


Figure 4. Tissue and chromatin-specific distribution of mussel macroH2A. A) Upper gel, SDS-PAGE of HCl-extracted proteins from different somatic tissues of the mussel *M. californianus* including: hepatopancreas (lane 1), muscle (lane 2), gills (lane 3) and hemolymph (lane 4). Lower gel, protein gel blot analysis of the HCl-extracted proteins using the invertebrate-specific anti-macroH2A antibody (M12) developed in the present work. B) Upper gel, SDS-PAGE of HCl-extracted proteins from different germinal tissues of the mussel *M. californianus* including: sperm (lane 1), male gonad (lane 2) and female gonad (lane 3). Lower gel, western blot analysis of the HCl-extracted proteins as in A). CM, chicken erythrocyte histones used as marker; rM, mussel recombinant macroH2A used as positive control; H4, anti-H4 antibodies used for sample normalization.

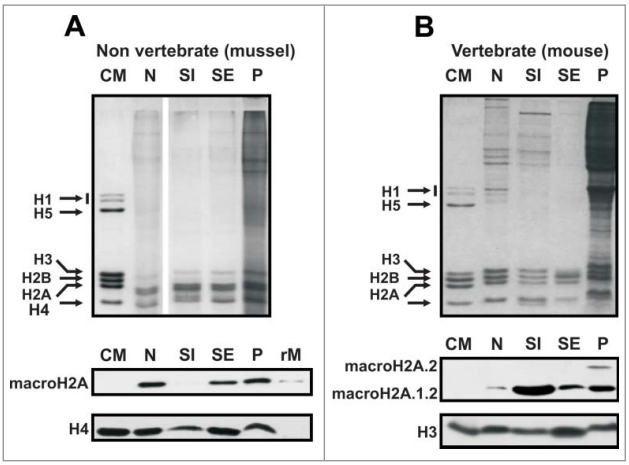


Figure 5. MacroH2A distribution in the chromatin of vertebrate and non-vertebrate organisms. A) Upper gel, SDS-PAGE analysis of the nuclear (N), SI, SE and P chromatin fractions obtained after digestion of mussel hepatopancreas nuclei with micrococcal nuclease. Lower gel, protein gel blot analysis of the HCl-extracted proteins as in A). B) Upper gel, SDS-PAGE analysis of the nuclear (N), SI, SE and P chromatin fractions obtained after digestion of mouse liver nuclei with micrococcal nuclease. Lower gel, Western blot analysis of the HCl-extracted proteins incubated with mouse-specific anti-macroH2A.1.2 and anti-macroH2A.2 antibodies (sequentially). CM, chicken erythrocyte histones used as marker; rM, mussel recombinant macroH2A used as positive control; H3 and H4, anti-H3 and anti-H4 antibodies used for sample normalization.

A new evolutionary framework for macroH2A

The identification of *Mg* macroH2A in mussels suggests that the origin of this variant is older than expected, probably arising before the split between protostomes and deuterostomes early in metazoan evolution. The present work corroborates such a hypothesis by providing conclusive evidence of 176 complete and non-redundant macroH2A sequences, including 144 from deuterostomes (phyla Echinodermata, Hemichordata, and Chordata), 22 from protostomes (Platyhelminthes, Rotifera, Arthropoda, Annelida, and Mollusca), 9 from basal Metazoans (Cnidarians, Placozoas, and Poriferans) and 1 from the Holozoan *Capsaspora* (Table S2). It is interesting to note that macroH2A sequences from invertebrates display conserved intron/exon structures, supporting their potential functionality

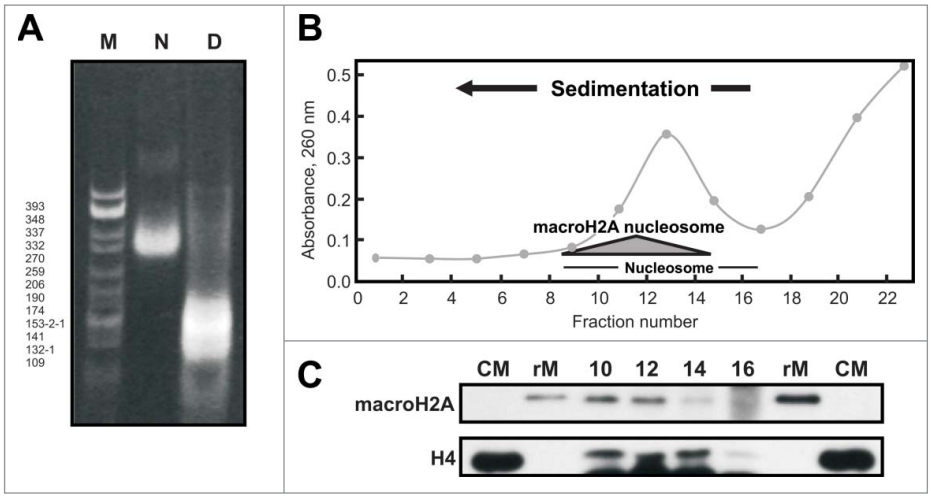


Figure 6. MacroH2A-nucleosomes in the mussel *Mytilus*. A) Native 4% (w/v) PAGE of the SI chromatin fraction obtained from *M. californianus* hepatopancreas. N, nucleosome; D, DNA; M, *Cfo*l-digested pBR322 plasmid DNA used as marker. B) Sucrose gradient fractionation of the SI fraction obtained from *M. californianus* hepatopancreas. The shaded triangle corresponds to the position of the nucleosomes containing macroH2A. C) Western blot analysis of the gradient fractions 10–16 using the invertebrate-specific anti-macroH2A antibody (M12) developed in the present work and anti-H4 antibody. Mussel recombinant macroH2A (rM) was used as positive control. CM, chicken erythrocyte histones used as marker.

(Fig. S2). Additionally, while partial macroH2As were retrieved from additional protostome groups such as Ctenophores and Tardigrades, this variant is apparently absent in nematodes and some insect groups.

The new catalog of macroH2A genes provided the basis for re-evaluating the evolutionary history of this lineage within the H2A family. Accordingly, molecular phylogenetic analyses support a monophyletic origin for the macroH2A lineage [36,38] as early as before the diversification of Filozoans (including Filasterea, Choanoflagellata, and Metazoa) as suggested by the presence of macroH2A in *Capsaspora* (Fig. 2, Fig. S3). The groups defined by the tree topology allowed us to clarify the identity of 9 uncertain macroH2A sequences as either macroH2A.1 or macroH2A.2. Both vertebrate variants constitute independent monophyletic groups, mirroring the functional constraints governing their evolution. Contrary to the existence of 2 vertebrate macroH2A variants (and 2 splicing forms for macroH2A.1), the reconstructed tree revealed the presence of a single macroH2A in invertebrates, similar to the case of histone variant H2A.Z.[39]

In vivo *identification of mussel histone macroH2A*

In order to take the study of macroH2A to a functional level, an anti-*Mg* macroH2A antibody (M12, Fig. S1) was developed in house. This antibody was used in Western blot experiments of histone extracts from species belonging to different non-vertebrate groups including cephalochordates, echinoderms, arthropods, molluscs, and cnidarians. In all instances, M12 recognized an electrophoretic band with a mobility corresponding to macroH2A (Fig. 3; see Fig. S4 for full blots). The small differences in electrophoretic mobility observed in most instances, most likely reflect compositional and/or conformational differences, as the sizes of the proteins predicted from their genomic sequences are very similar. The reason for the more pronounced change in electrophoretic mobility observed in the case of the sea urchin male gonad (Fig. 3, lane 3, Western) is a bit surprising and remains unclear at the present time. Altogether, this experiment showcased 3 major features of the M12 antibody: a) it is able to detect *Mg* macroH2A; b) it is also able to detect macroH2A in a wide range of non-vertebrate organisms; c) it is specific from non-vertebrates (i.e., it does not cross-react with vertebrate macroH2A).

The availability of the M12 antibody facilitated further functional analyses of this histone variant. Accordingly, macroH2A expression was studied in different tissues (including somatic and germinal) from non-vertebrate organisms and compared with the distribution of its vertebrate counterparts (Fig. 4A, 4B). The obtained results revealed the presence of macroH2A in all tissues studied with the exception of sperm. This variant was most abundant in hepatopancreas (functions equivalent to mammalian liver and pancreas) and gonadal tissue, followed by muscle and gills, and finally by a low presence in the hemolymph (function equivalent to vertebrate blood). In germinal tissues, this macroH2A was predominantly circumscribed to male and (in a lesser extent) to female gonadal tissues. Its complete absence in mature sperm is noticeable, especially since the sperm of *Mytilus*, in addition to its characteristic protamine-like proteins,[40] retains approximately 20–25% of histones, among which variants are included.[38,41,42]

Distribution and nucleosome organization of macroH2A in mussel chromatin

In order to further understand the functional role of macroH2A in mussel, its chromatin distribution was compared with that displayed by macroH2A in the mouse (vertebrate). Despite a very similar DNA partitioning in both organisms, the obtained results in mussel hepatopancreas show that macroH2A displays a predominant heterochromatin association with fractions SE and P and is present in low amounts in the fraction SI (euchromatic regions, Fig. 5A). Although macroH2A.1.2 is also distributed in SE and P fractions in mouse liver, a large amount of this histone is also distributed in the nuclease sensitive fraction (SI). This observation contrasts with the apparently limited distribution of mouse macroH2A.2 in

the P fraction, thus exhibiting a more similar pattern to that observed in mussel hepatopancreas (Fig. 5B).

Although *Mg* macroH2A displayed a low hybridization signal at the SI fraction (Fig. 5A), the high prevalence of mononucleosomes in this fraction provides an experimental basis to evaluate the recruitment of *Mg* macroH2A into nucleosomes.[43] Consequently, this sample was further fractionated using a sucrose gradient (Fig. 6A, 6B), taking advantage of the high DNA content of this fraction (approximately 35% of the total nuclear content before micrococcal nuclease digestion). The samples collected along the gradient were analyzed in western blot experiments using the invertebrate-specific anti-macroH2A antibody (M12) developed in the present work (Fig. 6C). Overall, the experimental results support the organization of *Mg* macroH2A into nucleosomes that, under the low ionic strength buffer conditions used in the sucrose gradient, run with a similar sedimentation coefficient as that of the canonical nucleosomes.[26]

Discussion

A new perspective on histone macroH2A origin and evolution

MacroH2A is probably the most enigmatic histone variant. Evolutionarily, it was widely accepted that this variant was circumscribed to vertebrates, contributing to the specialization in chromatin structure and epigenetic regulatory mechanisms through a subfunctionalization into macroH2A.1 and macroH2A.2.[26,44] The present work challenges this notion by characterizing, for the first time, a macroH2A variant in non-vertebrates, as well as the widespread distribution of this variant across metazoan animals. While macroH2A sequences from non-vertebrate organisms have been included previously as part of H2A phylogenies in papers reviewing histones, their nomenclature and role in epigenetics,[33,34,45] these reports do not provide molecular or biochemical experimental evidence (beyond *in silico* analyses of molecular databases) supporting the actual existence of these genes or their protein products *in vivo*. Therefore, we believe our results constitute the first experimental evidence supporting the presence of functional macroH2A beyond the vertebrate realm.

The characterization of *Mg* macroH2A revealed a gene structure displaying a considerable number of introns (8), similar to the case of its vertebrate counterpart (9 introns in human [5]). The nucleotide and protein sequence similarity of *Mg* macroH2A with its human counterpart (*Hs* macroH2A) mirrors its functional differentiation from the canonical H2A histone. However, contrary to the high overall similarity observed in the H2A domain and the macro domain, the L1 loop domain of macroH2A constitutes the most divergent region between both organisms. Since modifications within this domain might account for major structural and biochemical differences between canonical and macroH2A nucleosomes,[31] it would be of interest to investigate the consequences of such variation on the structure of *Mg* macroH2A-containing nucleosomes.[46,47]

The macroH2A lineage seems to have derived from a monophyletic origin during the evolution of the H2A family (Fig. 2), with macro and H2A domains remaining essentially conserved, probably as a result of the structural role of the H2A domain in the assembly of the nucleosome structure.[3] The presence of macroH2A in metazoan animals and in the Holozoan *Capsaspora owczarzaki* suggests that this variant originated before the radiation of Filozoans (including Filasterea, Choanoflagellata, and Metazoa), therefore being much older than previously thought. The presence of macroH2A in *Capsaspora* is especially interesting for 2 reasons: first, the genome of this unicellular organism has been shown to contain a significant number of genes related with metazoan multicellularity [48,49]; second, it has been documented that macroH2A regulates transcription of genes involved in vertebrate cell-cell signaling.[10] Altogether, although this variant might be in fact involved in many other processes, it is tempting to speculate that the presence of macroH2A in this organism could have played a relevant role during the eukaryotic transition toward multicellularity.

The conservation and widespread distribution of macroH2A across vertebrate and non-vertebrate organisms also supports its functional relevance during the evolution of metazoan animals. Such a notion is further reinforced by its specialization into macroH2A.1 and macroH2A.2,[5] a process reminiscent to that observed in the H2A.Z variant (e.g., H2A.Z.1 and H2A.Z.2), another monophyletic lineage within H2A.[39,50,51] In both cases, the differentiation of specialized vertebrate variants is concomitant with an increase in organismal complexity, underscoring the role of these variants in refining chromatin structure and the associated epigenetic mechanisms. On the contrary, the apparent absence of macroH2A in some protostome groups (i.e., nematodes and holometabolous insects) indicates that this variant might not be indispensable for the correct development of all organisms. While such observation agrees with the lack of apparent major dysfunctionality in macroH2A.1 and macroH2A.2 knockout mice,[17] it is in sharp contrast with other studies suggesting that this variant is essential for proper embryonic development.[21]

A functional perspective on macroH2A outside vertebrates

Although macroH2A is conserved across metazoans, the observed differences were enough to design an anti-macroH2A antibody specific for non-vertebrate organisms (M12, Fig. S1). Surprisingly, this antibody cross-reacts with histone H1 (Fig. S4), a phenomenon also observed in mouse specific anti-macroH2A antibodies.[15] While the significance of such cross-reactivity is still unknown, it would be tempting to speculate that it arises from the compositional similarity between the C-terminal domain of histone H1 and linker domain of macroH2A.[29,30] Interestingly, it has been shown that chromatin domains containing macroH2A nucleosomes are significantly depleted in H1.[26]

Protein expression experiments (Figs. 3–4) revealed the presence of macroH2A in all tissues studied in non-vertebrate organisms except for sperm, similarly to that observed for vertebrate macroH2A.[26,52-54] Interestingly, the absence of *Mg* macroH2A contrasts with the presence of other

variants in mussel sperm, including *Mg* H2A.X and *Mg* H2A.Z.[38] Overall, these results seem indicative of an exclusion of macroH2A from the chromatin of terminally differentiated cells in non-vertebrates.[26] The low amount of *Mg* macroH2A observed in hemolymph is consistent with this notion, as recent studies suggest that progenitor cells differentiate into hemocytes in the gills [55] reaching the hemolymph in a highly differentiated state. The elevated content of macroH2A in male and female gonadal tissue from non-vertebrate organisms also mirrors the distribution of macroH2A.1.2 in mouse male gonad.[15] This result is consistent with a role for this variant in cellular differentiation [15,19,21,26,56-58] and in meiosis, [26,59] which is further supported by the presence of *Mg* macroH2A in gills (a tissue involved in hematopoiesis [55]). Lastly, the high content of macroH2A in mussel hepatopancreas (liver) is also consistent with the association of vertebrate macroH2A.1 with liver and gonad,[15,26] underscoring its involvement in the regulation of vertebrate lipid metabolism.[11,17,22] Altogether, the distribution of macroH2A in non-vertebrates parallels that of vertebrate macroH2A.1 and departs from that of macroH2A.2, predominantly present in kidney and significantly less abundant in liver.[5]

Structurally, the association between macroH2A and heterochromatic regions[4-7,9,10,60] seems to extend also beyond vertebrates, as suggested by its presence in heterochromatin-containing fractions SE and P from mussel (Fig. 5). However, it remains to be elucidated whether macroH2A can or cannot act as context dependent transcriptional regulator in this group, similarly to what has been recently reported for macroH2A in mammals.[61] Indeed, mammalian macroH2A.1-containing chromatin domains have been reported to invade the transcribed regions of genes involved in development and cell-cell signaling.[10] Yet, the possibility remains that this later function was acquired during the subfunctionalization leading to the differentiation of macroH2A.1 and macroH2A.2 in vertebrates. Accordingly, macroH2A.2 might have retained a predominant heterochromatic involvement, whereas the 2 macroH2A.1 isoforms (at least macroH2A1.2) might have acquired a more ambivalent regulatory role.

Conclusions

The results presented in this work suggest that macroH2A is not an exclusive refinement of vertebrate chromatin. This conclusion challenges the notion suggesting that histone variants evolved from canonical histones. Instead, it supports a conserved ancestral role for histone variant lineages, based on the widespread presence of macroH2A and other specialized variants across metazoan animals.[33-35,45,46,62,63] Under this hypothesis, canonical histones (multiple copy, intronless, non polyadenylated genes) would have derived from ancestral variants (single copy, introns, polyadenylated genes) in order to produce enough protein product during the S-phase of the cell cycle to accommodate the newly synthesized DNA into nucleosomes. Overall, the results presented in this work help to configure a new evolutionary scenario in which histone variants, rather than modern "deviants" of canonical histones, would constitute ancient components of eukaryotic chromatin.

Material and methods

Identification of the macroH2A gene in the mussel Mytilus galloprovincialis

Total RNA was extracted from male gonad from mussel using Trizol (Invitrogen, Carlsbad, CA), and rapid amplification of 3′ and 5′ cDNA ends (RACE) was performed using the SMARTer RACE cDNA amplification Kit from 1 μg of total RNA in the presence of 100 U of the *SMARTScribe Reverse Transcriptase*, following the manufacturer's instructions (Clontech, Mountain View, CA). The obtained cDNAs were used to generate the 5′-RACE and 3′-RACE fragments in standard PCR reactions, using specific primers (mH2A_Fw1, mH2A_Fw2, mH2A_Rv1 and mH2A_Rv2) based on the partial macroH2A sequences identified through BLAST in *M. galloprovincialis* (CHROMEVALOA [64] accession numbers: NORM_MGT_c10112 and NORM_MGC_c4031, see Table S3). Briefly, 40 ng of cDNA were incubated with a mix containing: 0.2 μM primers, 1.5 mM $MgCl_2$ and 0.4 mM dNTPs, in presence of 1 U of *Taq* DNA polymerase enzyme (Roche Applied Science, Penzberg, Germany). Additional primers (mH2A_Full_Fw and mH2A_Full_Rv) were designed based on RACE sequences in order to obtain the complete mussel macroH2A cDNA sequence in a single reaction (see Table S3). The agarose gel-purified PCR product was ligated into *yT&A*^{TM} *Cloning Vector* and transformed into *ECOS*^{TM} *9–5* competent cells (Yeastern Biotech Co., Taiwan, China). Plasmid DNA was obtained from these cells using the QIAprep Spin Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and subsequently sequenced in a 3130xl sequencer (Applied Biosystems, Foster City, CA). DNA was sequenced using a CEQ8000 sequencer (Beckman Coulter Inc., Brea, CA). The obtained sequence (*Mg* macroH2A) was deposited in the GenBank database under the accession number KT894822.

Phylogenetic inference

A total of 176 macroH2A sequences were retrieved from molecular databases (histone database [65] and GenBank [66] as of April 2015), including 144 from deuterostomes (phyla Echinodermata, Hemichordata, and Chordata), 22 from protostomes (Platyhelminthes, Rotifera, Arthropoda, Annelida, and Mollusca), 9 from basal Metazoans (Cnidarians, Placozoas, and Poriferans) and 1 from the Holozoan *Capsaspora* (Table S2). Partial macroH2As were also found in Ctenophores and Tardigrades. Sequences were aligned using the BioEdit program[67], molecular evolutionary analyses were conducted using the program MEGA version 6,[68] and a maximum likelihood (ML) macroH2A phylogeny was reconstructed based on the H2A domain using the JTT substitution model[69] with gamma-distributed variation across sites. The reliability of the tree topology was contrasted using bootstrap analysis (1,000 replicates) and human histone H2A.Z was used as the outgroup.[39,50]

Histone extraction and recombinant macroH2A from Mytilus

Protein extractions were performed according to [70] from different tissues of several non-vertebrate groups, including: gills, male and female gonads, sperm, hepatopancreas, hemolymph and muscle from the mussel *Mytilus californianus*; larval tissue from the sea anemone *Nematostella vectensis*; salivary glands from the tick *Amblyomma maculatum*; male gonad from the sea urchin *Strongylocentrotus purpuratus*; whole body extract from amphioxus *Branchiostoma floridae*; and liver tissue from the mouse *Mus musculus*. Tissues were homogenized in approximately 5 volumes of 0.15 M NaCl, 10 mM Tris-HCl (pH 7.5), 0.5% (v/v) Triton X-100 buffer containing Roche Complete Protease Cocktail inhibitor (Roche Molecular Biochemicals, Laval, QC; one tablet per 100 ml) using a Dounce homogenizer. Homogenates were incubated at 4°C for 10 min and subsequently centrifuged at 4,000 × g in Eppendorf tubes. Pellets were re-suspended in 0.1 M KCl, 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, incubated at 4°C for 10 min and centrifuged again at 4,000 × g. The nuclear pellets thus obtained were homogenized in approximately 3 volumes of 0.6 N HCl and centrifuged at 8,200 × g. The supernatant extracts were precipitated with 6 volumes of acetone at −20°C overnight and then centrifuged at 10,000 × g for 10 min at 4°C. The acetone pellets were dried using a Speedvac concentrator and stored at −80°C until further use. Recombinant macroH2A was obtained as described elsewhere.[26]

Chromatin fractionation

Chromatin from mouse liver and mussel hepatopancreas were digested with micrococcal nuclease. In this very coarse fractionation,[71,72] the SI (supernatant) fraction recovered immediately upon nuclease digestion contains digested DNA and nucleosomes from chromatin regions readily accessible to the nuclease (i.e., euchromatic regions). The SI fraction was subsequently fractionated further using a 5–20% sucrose gradient in 25 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.2 mM EDTA buffer run for 21 h at 96,000 × g at 4°C. The SE (supernatant) fraction, obtained after hypotonic lysis of the pelleted nuclei, is highly enriched in facultative heterochromatin. Lastly, the P (pellet) fraction is highly heterogeneous and consists of more nuclease resilient constitutive heterochromatin and insoluble transcription factor co-activator-containing transcriptional assembly complexes from the promoter regions of active genes.[71]

Preparation of anti-macroH2A antibodies specific for non-vertebrates

MacroH2A protein sequences were aligned (see Table S2) and 2 peptides corresponding to conserved regions different from vertebrate macroH2A counterparts were synthesized and KLH coupled by GL Biochem (Shanghai) Ltd. (China): LSEKKLFLGQKM (peptide M1), located at the NHD region, and GGVLPHIHPELL (peptide M2), located at the H2A domain of macroH2A (Fig. S1). Peptides M1 and a mixture of the 2 (M1+M2) were injected in 2 different rabbits at the animal care facility unit of the University of Victoria (Canada). Antibody specificity was tested by means of ELISA and Western blot, using a recombinant macroH2A protein from the mussel *Mytilus* (see above). The sera obtained from both rabbits specifically recognized macroH2A in non-vertebrates, but not the vertebrate counterpart. The second antibody (M1+M2) was used in this work due to its higher immunoreactivity.

Polyacrylamide gel electrophoresis (PAGE) and protein gel blot experiments

Fifteen per cent (w/v) polyacrylamide SDS gels[38] and native 4% (w/v) PAGE were prepared and run as described elsewhere.[38] The SDS-PAGE gels loaded with approximately 2 μg of histones were transferred onto nitrocellulose membranes at 100 V for 3 h at 4°C in 20 mM sodium phosphate (pH 6.8), 14.25% (v/v) ethanol and 0.1% (w/v) SDS transfer buffer. The membranes were blocked in PBS, 0.1% (v/v) Tween and 3% (w/v) skimmed milk at room temperature for 1 h and subsequently incubated with the primary antibody, diluted in blocking buffer, at 4°C overnight. Primary antibodies were used at the following dilutions: M12 non-vertebrate macroH2A (synthesized in the present work), 1:1,000; vertebrate macroH2A.1.2, 1:2,000 (Abcam, Cambridge, UK); vertebrate macroH2A.2, 1:500 (Abcam, Cambridge, UK); H4 histone (prepared in house), 1:10,000; and H3 histone, 1:10,000 (Sigma-Aldrich, St. Louis, MO). After incubation with the primary antibody, membranes were washed 4 times, 10 min at room temperature in PBS and 0.1% Tween. Membranes were then incubated with the secondary antibody [ECL rabbit IgG, HRP-linked whole Ab (GE Healthcare, Piscataway, NJ)] for 1 h at room temperature and at a 1:5,000 dilution (or 1:2,000 dilution for macroH2A). The secondary antibody signal was detected using Luminata Forte western HRP (Millipore, Billerica, MA).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Shahid Karim at the Department of Biological Sciences of the University of Southern Mississippi for providing us with salivary glands from the tick *Amblyomma maculatum*, Nancy Sherwood from the Biology Department at the University of Victoria for providing us with several specimens of the lancelet *Branchiostoma floridae*, and Robert Burke from the Biochemistry and Microbiology Department at the University of Victoria for providing us with tissue samples of the sea urchin *Strongylocentrotus purpuratus*. We are grateful to Angel Vizoso-Vazquez, Esperanza Cerdan and Josefina Mendez at the Department of Cellular and Molecular Biology (University of A Coruna, Spain) for providing CR-C with lab space and support during macroH2A purification. We are also very thankful to Frederic Berger, Juan Pedro Martinez Camacho, Steven Henikoff and Paul Talbert, as well as to the colleagues attending the 2015 Gordon Conference on Epigenetics, for their comments and feedback on an earlier version of this manuscript. This is contribution #1 of the Marine Education and Research Center of the Institute for Water and the Environment at Florida International University.

Funding

This work was supported by grants from the Biomolecular Sciences Institute (800005997) and the College of Arts, Sciences and Education (CASE) at Florida International University (JME-L); and by a Natural Sciences and Engineering Research Council of Canada (NSERC) 46399-2012 grant to JA. CR-C was supported by an FPU fellowship from the Government of Spain.

References

1. Bonisch C, Hake SB. Histone H2A variants in nucleosomes and chromatin: more or less stable? Nucleic Acids Res 2012; 40:10719-41; PMID:23002134; http://dx.doi.org/10.1093/nar/gks865
2. Ausió J, Abbott DW. The many tales of a tail: carboxyl-terminal tail heterogeneity specializes histone H2A variants for defined chromatin function. Biochemistry 2002; 41:5945-9; PMID:11993987; http://dx.doi.org/10.1021/bi020059d
3. Pehrson JR, Fried VA. MacroH2A, a core histone containing a large nonhistone region. Science 1992; 257:1398-400; PMID:1529340; http://dx.doi.org/10.1126/science.1529340
4. Costanzi C, Pehrson JR. Histone macroH2A1 is concentrated in the inactive X chromosome of female mammals. Nature 1998; 393:599-601; PMID:9634239; http://dx.doi.org/10.1038/31275
5. Costanzi C, Pehrson JR. MACROH2A2, a new member of the MARCOH2A core histone family. J Biol Chem 2001; 276:21776-84; PMID:11262398; http://dx.doi.org/10.1074/jbc.M010919200
6. Richler C, Dhara SK, Wahrman J. Histone macroH2A1.2 is concentrated in the XY compartment of mammalian male meiotic nuclei. Cytogenet Cell Genet 2000; 89:118-20; PMID:10894952; http://dx.doi.org/10.1159/000015589
7. Rasmussen TP, Mastrangelo MA, Eden A, Pehrson JR, Jaenisch R. Dynamic relocalization of histone MacroH2A1 from centrosomes to inactive X chromosomes during X inactivation. J Cell Biol 2000; 150:1189-98; PMID:10974005; http://dx.doi.org/10.1083/jcb.150.5.1189
8. Mermoud JE, Costanzi C, Pehrson JR, Brockdorff N. Histone macroH2A1.2 relocates to the inactive X chromosome after initiation and propagation of X-inactivation. J Cell Biol 1999; 147:1399-408; PMID:10613899; http://dx.doi.org/10.1083/jcb.147.7.1399
9. Zhang R, Poustovoitov MV, Ye X, Santos HA, Chen W, Daganzo SM, Erzberger JP, Serebriiskii IG, Canutescu AA, Dunbrack RL, et al. Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. Dev Cell 2005; 8:19-30; PMID:15621527; http://dx.doi.org/10.1016/j.devcel.2004.10.019
10. Gamble MJ, Frizzell KM, Yang C, Krishnakumar R, Kraus WL. The histone variant macroH2A1 marks repressed autosomal chromatin, but protects a subset of its target genes from silencing. Genes Dev 2010; 24:21-32; PMID:20008927; http://dx.doi.org/10.1101/gad.1876110
11. Changolkar LN, Singh G, Cui K, Berletch JB, Zhao K, Disteche CM, Pehrson JR. Genome-wide distribution of macroH2A1 histone variants in mouse liver chromatin. Mol Cell Biol 2010; 30:5473-83; PMID:20937776; http://dx.doi.org/10.1128/MCB.00518-10
12. Ouararhni K, Hadj-Slimane R, Ait-Si-Ali S, Robin P, Mietton F, Harel-Bellan A, Dimitrov S, Hamiche A. The histone variant mH2A1.1 interferes with transcription by down-regulating PARP-1 enzymatic activity. Genes Dev 2006; 20:3324-36; PMID:17158748; http://dx.doi.org/10.1101/gad.396106
13. Creppe C, Posavec M, Douet J, Buschbeck M. MacroH2A in stem cells: a story beyond gene repression. Epigenomics 2012; 4:221-7; PMID:22449192; http://dx.doi.org/10.2217/epi.12.8
14. Rasmussen TP, Huang T, Mastrangelo MA, Loring J, Panning B, Jaenisch R. Messenger RNAs encoding mouse histone macroH2A1 isoforms are expressed at similar levels in male and female cells and result from alternative splicing. Nucleic Acids Res 1999; 27:3685-9; PMID:10471737; http://dx.doi.org/10.1093/nar/27.18.3685
15. Pehrson JR, Costanzi C, Dharia C. Developmental and tissue expression patterns of histone macroH2A1 subtypes. J Cell Biochem 1997; 65:107-13; PMID:9138085; http://dx.doi.org/10.1002/(SICI)1097-4644(199704)65:1%3c107::AID-JCB11%3e3.0.CO;2-H
16. Araya I, Nardocci G, Morales J, Vera M, Molina A, Alvarez M. MacroH2A subtypes contribute antagonistically to the transcriptional regulation of the ribosomal cistron during seasonal acclimatization of the carp fish. Epigenetics Chromatin 2010; 3:14; PMID:20670405; http://dx.doi.org/10.1186/1756-8935-3-14
17. Pehrson JR, Changolkar LN, Costanzi C, Leu NA. Mice without macroH2A histone variants. Mol Cell Biol 2014; 34:4523-33; PMID:25312643; http://dx.doi.org/10.1128/MCB.00794-14
18. Chang CC, Gao S, Sung LY, Corry GN, Ma Y, Nagy ZP, Tian XC, Rasmussen TP. Rapid elimination of the histone variant MacroH2A from somatic cell heterochromatin after nuclear transfer. Cell Reprogram 2010; 12:43-53; PMID:20132012; http://dx.doi.org/10.1089/cell.2009.0043
19. Creppe C, Janich P, Cantarino N, Noguera M, Valero V, Musulen E, Douet J, Posavec M, Martin-Caballero J, Sumoy L, et al. MacroH2A1 regulates the balance between self-renewal and differentiation commitment in embryonic and adult stem cells. Mol Cell Biol 2012; 32:1442-52; PMID:22331466; http://dx.doi.org/10.1128/MCB.06323-11
20. Gaspar-Maia A, Qadeer ZA, Hasson D, Ratnakumar K, Leu NA, Leroy G, Liu S, Costanzi C, Valle-Garcia D, Schaniel C, et al. MacroH2A histone variants act as a barrier upon reprogramming towards pluripotency. Nat Commun 2013; 4:1565; PMID:23463008; http://dx.doi.org/10.1038/ncomms2582
21. Buschbeck M, Uribesalgo I, Wibowo I, Rue P, Martin D, Gutierrez A, Morey L, Guigo R, Lopez-Schier H, Di Croce L. The histone variant macroH2A is an epigenetic regulator of key developmental genes. Nat Struct Mol Biol 2009; 16:1074-9; PMID:19734898; http://dx.doi.org/10.1038/nsmb.1665
22. Changolkar LN, Costanzi C, Leu NA, Chen D, McLaughlin KJ, Pehrson JR. Developmental changes in histone macroH2A1-mediated gene regulation. Mol Cell Biol 2007; 27:2758-64; PMID:17242180; http://dx.doi.org/10.1128/MCB.02334-06
23. Monteiro FL, Baptista T, Amado F, Vitorino R, Jeronimo C, Helguero LA. Expression and functionality of histone H2A variants in cancer. Oncotarget 2014; 5:3428-43; PMID:25003966; http://dx.doi.org/10.18632/oncotarget.2007
24. Dardenne E, Pierredon S, Driouch K, Gratadou L, Lacroix-Triki M, Espinoza MP, Zonta E, Germann S, Mortada H, Villemin JP, et al. Splicing switch of an epigenetic regulator by RNA helicases promotes tumor-cell invasiveness. Nat Struct Mol Biol 2012; 19:1139-46; PMID:23022728; http://dx.doi.org/10.1038/nsmb.2390
25. Novikov L, Park JW, Chen H, Klerman H, Jalloh AS, Gamble MJ. QKI-mediated alternative splicing of the histone variant MacroH2A1 regulates cancer cell proliferation. Mol Cell Biol 2011; 31:4244-55; PMID:21844227; http://dx.doi.org/10.1128/MCB.05244-11
26. Abbott DW, Laszczak M, Lewis JD, Su H, Moore SC, Hills M, Dimitrov S, Ausio J. Structural characterization of macroH2A containing chromatin. Biochemistry 2004; 43:1352-9; PMID:14756572; http://dx.doi.org/10.1021/bi035859i
27. Chakravarthy S, Luger K. The histone variant macro-H2A preferentially forms "hybrid nucleosomes". J Biol Chem 2006; 281:25522-31; PMID:16803903; http://dx.doi.org/10.1074/jbc.M602258200
28. Angelov D, Molla A, Perche PY, Hans F, Cote J, Khochbin S, Bouvet P, Dimitrov S. The histone variant macroH2A interferes with transcription factor binding and SWI/SNF nucleosome remodeling. Mol Cell 2003; 11:1033-41; PMID:12718888; http://dx.doi.org/10.1016/S1097-2765(03)00100-X
29. Muthurajan UM, McBryant SJ, Lu X, Hansen JC, Luger K. The linker region of macroH2A promotes self-association of nucleosomal arrays. J Biol Chem 2011; 286:23852-64; PMID:21532035; http://dx.doi.org/10.1074/jbc.M111.244871
30. Chakravarthy S, Patel A, Bowman GD. The basic linker of macroH2A stabilizes DNA at the entry/exit site of the nucleosome. Nucleic Acids Res 2012; 40:8285-95; PMID:22753032; http://dx.doi.org/10.1093/nar/gks645
31. Chakravarthy S, Gundimella SK, Caron C, Perche PY, Pehrson JR, Khochbin S, Luger K. Structural characterization of the histone variant macroH2A. Mol Cell Biol 2005; 25:7616-24; PMID:16107708; http://dx.doi.org/10.1128/MCB.25.17.7616-7624.2005

32. Nusinow DA, Hernandez-Munoz I, Fazzio TG, Shah GM, Kraus WL, Panning B. Poly(ADP-ribose) polymerase 1 is inhibited by a histone H2A variant, MacroH2A, and contributes to silencing of the inactive X chromosome. J Biol Chem 2007; 282:12851-9; PMID:17322296; http://dx.doi.org/10.1074/jbc.M610502200
33. Henikoff S, Smith MM. Histone variants and epigenetics. Cold Spring Harb Perspect Biol 2015; 7:a019364; PMID:25561719; http://dx.doi.org/10.1101/cshperspect.a019364
34. Talbert PB, Ahmad K, Almouzni G, Ausio J, Berger F, Bhalla PL, Bonner WM, Cande WZ, Chadwick BP, Chan SW, et al. A unified phylogeny-based nomenclature for histone variants. Epigenetics Chromatin 2012; 5:7; PMID:22650316; http://dx.doi.org/10.1186/1756-8935-5-7
35. Eirín-López JM, González-Romero R, Dryhurst D, Méndez J, Ausió J. Long-term evolution of histone families: old notions and new insights into their diversification mechanisms across eukaryotes. In: Pontarotti P, ed. Evolutionary Biology: Concept, Modeling, and Application. Berlin Heidelberg: Springer-Verlag, 2009:139-62; http://dx.doi.org/10.1007/978-3-642-00952-5_8
36. Gonzalez-Romero R, Ausio J, Mendez J, Eirin-Lopez JM. Early evolution of histone genes: prevalence of an 'orphon' H1 lineage in protostomes and birth-and-death process in the H2A family. J Mol Evol 2008; 66:505-18; PMID:18443735; http://dx.doi.org/10.1007/s00239-008-9109-1
37. Kasinsky H, Lewis J, Dacks J, Ausió J. Origin of H1 linker histones. FASEB J 2001; 15:34-42; PMID:11149891; http://dx.doi.org/10.1096/fj.00-0237rev
38. Gonzalez-Romero R, Rivera-Casas C, Frehlick LJ, Mendez J, Ausio J, Eirin-Lopez JM. Histone H2A (H2A.X and H2A.Z) variants in molluscs: molecular characterization and potential implications for chromatin dynamics. PLoS One 2012; 7:e30006; PMID:22253857; http://dx.doi.org/10.1371/journal.pone.0030006
39. Eirin-Lopez JM, Gonzalez-Romero R, Dryhurst D, Ishibashi T, Ausio J. The evolutionary differentiation of two histone H2A.Z variants in chordates (H2A.Z-1 and H2A.Z-2) is mediated by a stepwise mutation process that affects three amino acid residues. BMC Evol Biol 2009; 9:31; PMID:19193230; http://dx.doi.org/10.1186/1471-2148-9-31
40. Eirin-Lopez JM, Ausio J. Origin and evolution of chromosomal sperm proteins. Bioessays 2009; 31:1062-70; PMID:19708021; http://dx.doi.org/10.1002/bies.200900050
41. Lewis JD, Ausió J. Protamine-like proteins: evidence for a novel chromatin structure. Biochemistry and cell biology = Biochimie et biologie cellulaire 2002; 80:353-61; PMID:12123288; http://dx.doi.org/10.1139/o02-083
42. Avramova Z, Zalensky A, Tsanev R. Biochemical and ultrastructural study of the sperm chromatin from Mytilus galloprovincialis. Experimental cell research 1984; 152:231-9; PMID:6232144; http://dx.doi.org/10.1016/0014-4827(84)90248-9
43. Ishibashi T, Thambirajah AA, Ausio J. MeCP2 preferentially binds to methylated linker DNA in the absence of the terminal tail of histone H3 and independently of histone acetylation. FEBS letters 2008; 582:1157-62; PMID:18339321; http://dx.doi.org/10.1016/j.febslet.2008.03.005
44. Malik HS, Henikoff S. Phylogenomics of the nucleosome. Nat Struct Biol 2003; 10:882-91; PMID:14583738; http://dx.doi.org/10.1038/nsb996
45. Talbert PB, Henikoff S. Histone variants–ancient wrap artists of the epigenome. Nat Rev Mol Cell Biol 2010; 11:264-75; PMID:20197778; http://dx.doi.org/10.1038/nrm2861
46. Cheema MS, Ausio J. The Structural Determinants behind the Epigenetic Role of Histone Variants. Genes (Basel) 2015; 6:685-713; PMID:26213973; http://dx.doi.org/10.3390/genes6030685
47. Luger K, Mäder AW, Richmond RK, Sargent DF, Richmond TJ. Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 1997; 389:251-60; PMID:9305837; http://dx.doi.org/10.1038/38444
48. Ruiz-Trillo I, Roger AJ, Burger G, Gray MW, Lang BF. A phylogenomic investigation into the origin of metazoa. Mol Biol Evol 2008; 25:664-72; PMID:18184723; http://dx.doi.org/10.1093/molbev/msn006
49. Sebe-Pedros A, de Mendoza A, Lang BF, Degnan BM, Ruiz-Trillo I. Unexpected repertoire of metazoan transcription factors in the unicellular holozoan Capsaspora owczarzaki. Mol Biol Evol 2011; 28:1241-54; PMID:21087945; http://dx.doi.org/10.1093/molbev/msq309
50. Dryhurst D, Ishibashi T, Rose KL, Eirin-Lopez JM, McDonald D, Silva-Moreno B, Veldhoen N, Helbing CC, Hendzel MJ, Shabanowitz J, et al. Characterization of the histone H2A.Z-1 and H2A.Z-2 isoforms in vertebrates. BMC Biol 2009; 7:86; PMID:20003410; http://dx.doi.org/10.1186/1741-7007-7-86
51. Bonisch C, Schneider K, Punzeler S, Wiedemann SM, Bielmeier C, Bocola M, Eberl HC, Kuegel W, Neumann J, Kremmer E, et al. H2A.Z.2.2 is an alternatively spliced histone H2A.Z variant that causes severe nucleosome destabilization. Nucleic Acids Res 2012; 40(13):5951-64; PMID: 22467210; http://dx.doi.org/10.1093/nar/gks267
52. Turner JM, Mahadevaiah SK, Elliott DJ, Garchon HJ, Pehrson JR, Jaenisch R, Burgoyne PS. Meiotic sex chromosome inactivation in male mice with targeted disruptions of Xist. J Cell Sci 2002; 115:4097-105; PMID:12356914; http://dx.doi.org/10.1242/jcs.00111
53. Chang CC, Ma Y, Jacobs S, Tian XC, Yang X, Rasmussen TP. A maternal store of macroH2A is removed from pronuclei prior to onset of somatic macroH2A expression in preimplantation embryos. Dev Biol 2005; 278:367-80; PMID:15680357; http://dx.doi.org/10.1016/j.ydbio.2004.11.032
54. Castillo J, Amaral A, Azpiazu R, Vavouri T, Estanyol JM, Ballesca JL, Oliva R. Genomic and proteomic dissection and characterisation of the human sperm chromatin. Molecular human reproduction 2014; 20(11):1041-53; PMID:25193639; http://dx.doi.org/10.1093/molehr/gau079
55. Jemaa M, Morin N, Cavelier P, Cau J, Strub JM, Delsert C. Adult somatic progenitor cells and hematopoiesis in oysters. J Exp Biol 2014; 217:3067-77; PMID:24948634; http://dx.doi.org/10.1242/jeb.106575
56. Pasque V, Gillich A, Garrett N, Gurdon JB. Histone variant macroH2A confers resistance to nuclear reprogramming. Embo j 2011; 30:2373-87; PMID:21552206; http://dx.doi.org/10.1038/emboj.2011.144
57. Pasque V, Radzisheuskaya A, Gillich A, Halley-Stott RP, Panamarova M, Zernicka-Goetz M, Surani MA, Silva JC. Histone variant macroH2A marks embryonic differentiation in vivo and acts as an epigenetic barrier to induced pluripotency. J Cell Sci 2012; 125:6094-104; PMID:23077180; http://dx.doi.org/10.1242/jcs.113019
58. Barrero MJ, Sese B, Marti M, Izpisua Belmonte JC. Macro histone variants are critical for the differentiation of human pluripotent cells. J Biol Chem 2013; 288:16110-6; PMID:23595991; http://dx.doi.org/10.1074/jbc.M113.466144
59. Lewis JD, Abbott DW, Ausio J. A haploid affair: core histone transitions during spermatogenesis. Biochem Cell Biol 2003; 81:131-40; PMID:12897846; http://dx.doi.org/10.1139/o03-045
60. Mermoud JE, Tassin AM, Pehrson JR, Brockdorff N. Centrosomal association of histone macroH2A1.2 in embryonic stem cells and somatic cells. Exp Cell Res 2001; 268:245-51; PMID:11478850; http://dx.doi.org/10.1006/excr.2001.5277
61. Gamble MJ, Kraus WL. Multiple facets of the unique histone variant macroH2A: from genomics to cell biology. Cell Cycle 2010; 9:2568-74; PMID:20543561; http://dx.doi.org/10.4161/cc.9.13.12144
62. Maze I, Noh KM, Soshnev AA, Allis CD. Every amino acid matters: essential contributions of histone variants to mammalian development and disease. Nat Rev Genet 2014; 15:259-71; PMID:24614311; http://dx.doi.org/10.1038/nrg3673
63. Ausio J. Histone variants–the structure behind the function. Brief Funct Genomic Proteomic 2006; 5:228-43; PMID:16772274; http://dx.doi.org/10.1093/bfgp/ell020
64. Suarez-Ulloa V, Fernandez-Tajes J, Aguiar-Pulido V, Rivera-Casas C, Gonzalez-Romero R, Ausio J, Mendez J, Dorado J, Eirin-Lopez JM. The CHROMEVALOA database: a resource for the evaluation of Okadaic Acid contamination in the marine environment based on the chromatin-associated transcriptome of the mussel Mytilus galloprovincialis. Mar Drugs 2013; 11:830-41; PMID:23481679; http://dx.doi.org/10.3390/md11030830
65. Marino-Ramirez L, Levine KM, Morales M, Zhang S, Moreland RT, Baxevanis AD, Landsman D. The Histone Database: an integrated resource for histones and histone fold-containing proteins. Database (Oxford) 2011; 2011:bar048.
66. Benson DA, Cavanaugh M, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. GenBank. Nucleic Acids Res 2013; 41:D36-42; PMID:23193287; http://dx.doi.org/10.1093/nar/gks1195

67. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 1999; 41:95-8.
68. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 2013; 30:2725-9; PMID:24132122; http://dx.doi.org/10.1093/molbev/mst197
69. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. Comput Applic Biosci 1992; 8:275-82.
70. Ausió J, Moore SC. Reconstitution of chromatin complexes from high-performance liquid chromatography-purified histones. Methods (San Diego, Calif) 1998; 15:333-42; PMID:9740721; http://dx.doi.org/10.1006/meth.1998.0637
71. Ishibashi T, Dryhurst D, Rose KL, Shabanowitz J, Hunt DF, Ausio J. Acetylation of vertebrate H2A.Z and its effect on the structure of the nucleosome. Biochemistry 2009; 48:5007-17; PMID:19385636; http://dx.doi.org/10.1021/bi900196c
72. Thambirajah AA, Ng MK, Frehlick LJ, Li A, Serpa JJ, Petrotchenko EV, Silva-Moreno B, Missiaen KK, Borchers CH, Adam Hall J, et al. MeCP2 binds to nucleosome free (linker DNA) regions and to H3K9/H3K27 methylated nucleosomes in the brain. Nucleic acids research 2011; 40:2884-97; PMID:22144686; http://dx.doi.org/10.1093/nar/gkr1066
73. Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ. The Phyre2 web portal for protein modeling, prediction and analysis. Nature protocols 2015; 10:845-58; PMID:25950237; http://dx.doi.org/10.1038/nprot.2015.053