Higher concentrations of histone macroH2A in the Barr body are correlated with higher nucleosome density

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Histone macroH2A, which is a subtype of histone H2A, possesses a histone H2A-like portion fused to a relatively long non-histone portion. MacroH2A has been shown to associate preferentially with the inactive X chromosome [1]. To investigate the specificity of this association, the nuclear distribution of macroH2A was compared with that of regular core histones. In normal human female fibroblasts, all anti-histone antibodies that were tested (including anti-macroH2A antibody) preferentially labeled the inactive X chromosome. Moreover, when expressed as green fluorescent protein (GFP) fusions, both histone H2A and macroH2A were concentrated in the Barr body. These data clearly show the presence of a higher density of nucleosomes in the inactive X chromosome. Accordingly, the specificity of the macroH2A association with the inactive X chromosome should be reconsidered. While investigating the role of macroH2A, we found that the proximity of the non-histone region of macroH2A to a promoter could lead to a specific repression of transcription, suggesting that the incorporation of macroH2A into chromatin might help to establish the stable pattern of gene expression in differentiated cells.

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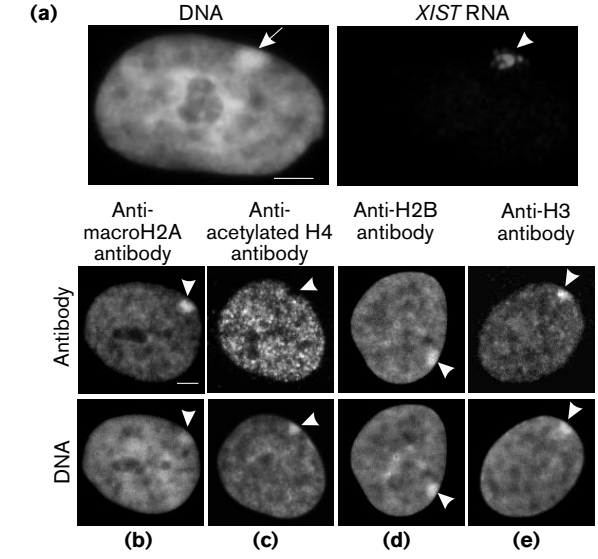
Results and discussion

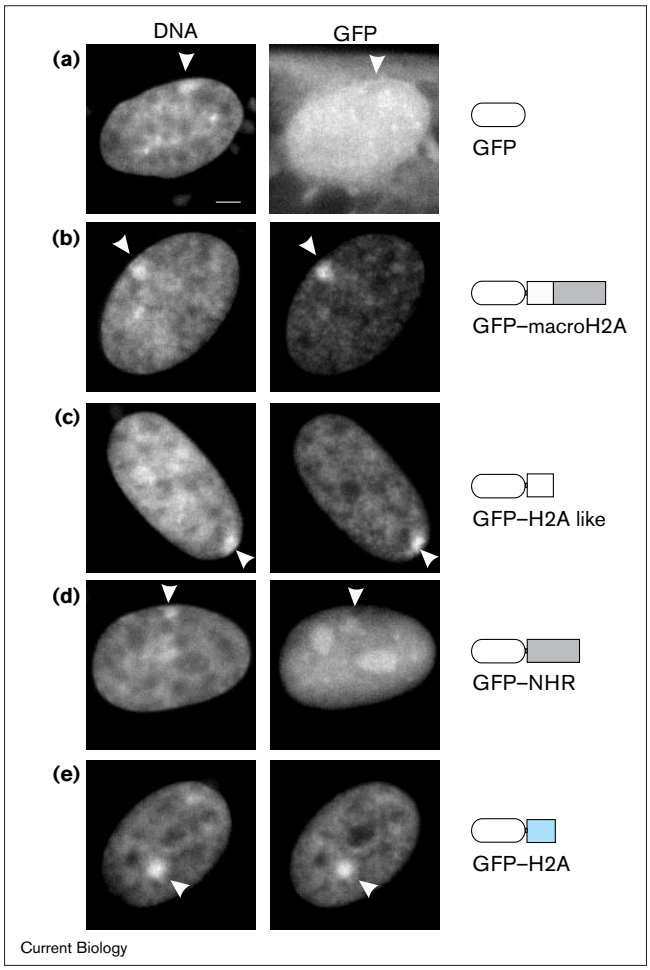
Normal human diploid female dermal fibroblasts [2] containing one inactive X chromosome were used to investigate the specific association of macroH2A and regular core histones with the inactive X chromosome. In the interphase nuclei of these cells, the Barr body could be visualized unambiguously after staining of DNA with the dye 4,6-diamidino-2-phenylindole (DAPI, Figure 1a). The identity of this structure was confirmed with an *XIST* RNA probe used in fluorescent *in situ* hybridization (FISH) [3] (Figure 1a). Figure 1b–e shows, as previously reported, the preferential labeling of the Barr body by an anti-macroH2A antibody (Figure 1b). As expected, this region was devoid of acetylated histone H4 [4] (Figure 1c). Surprisingly, anti-core histone antibodies also preferentially labeled the inactive X chromosome (Figure 1d,e). This labeling of the Barr body by the anti-histone antibodies could be due either to a better accessibility of histones to the antibodies or to a higher concentration of nucleosomes (denser chromatin structure). To distinguish

Figure 1



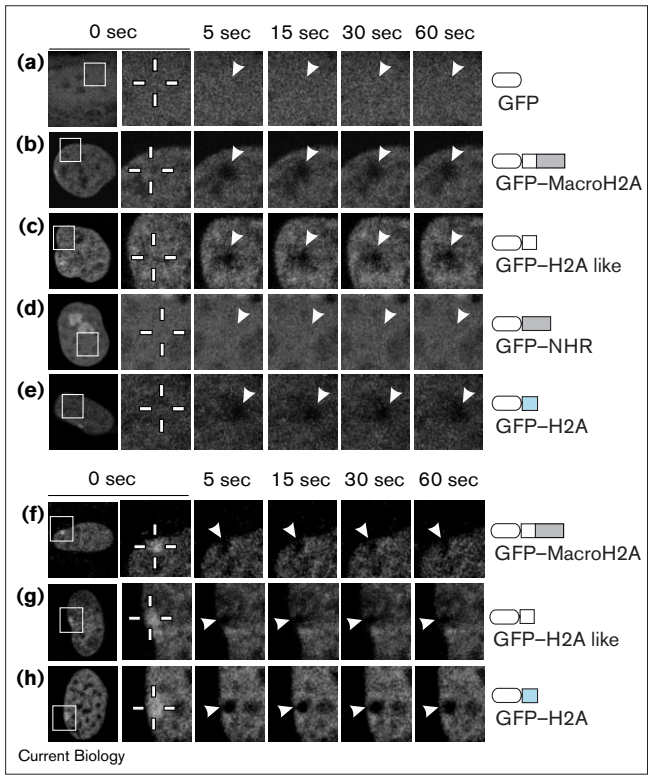
(a) Identification of the Barr body in normal human fibroblasts. Normal primary human dermal fibroblasts [2] were grown and fixed on LabTek glass slides. The inactive X chromosome was identified with an *XIST* RNA probe used in FISH [3] (right panel), and the nuclei counterstained with DAPI (left panel). The arrow points to the Barr body in a DAPI-stained nucleus. The arrowhead indicates the *XIST* RNA revealed by FISH. **(b–e)** Both core histones and macroH2A are concentrated in the Barr body. Normal human fibroblasts were fixed in 4% paraformaldehyde for 5 min at room temperature and the indicated antibodies were used to immunolocalize (b) macroH2A, (c) acetylated histone H4, (d) histone H2B and (e) histone H3. Arrowheads indicate the position of the Barr body. Anti-macroH2A antibody is a mouse ascitic fluid obtained in our laboratory after the injection of the bacterially expressed non-histone domain of human macroH2A1.2 in mice. Anti-H2B and anti-H3 antibodies were kindly provided by S. Muller. Anti-acetylated histone H4 was from a commercially available source. The scale bars represent 2.5 μm.

Figure 2



Specific targeting of the Barr body by GFP fusions with histones macroH2A and H2A. Normal human fibroblasts were transfected using Exgen reagent (Euromedex) and constructs expressing the indicated fusion proteins, according to the supplier's recommendations; 24 h post-transfection, cells were fixed and the GFP and DAPI fluorescence recorded. Arrowheads indicate the position of the Barr body. The scale bar represents 2.5 μm.

Figure 3

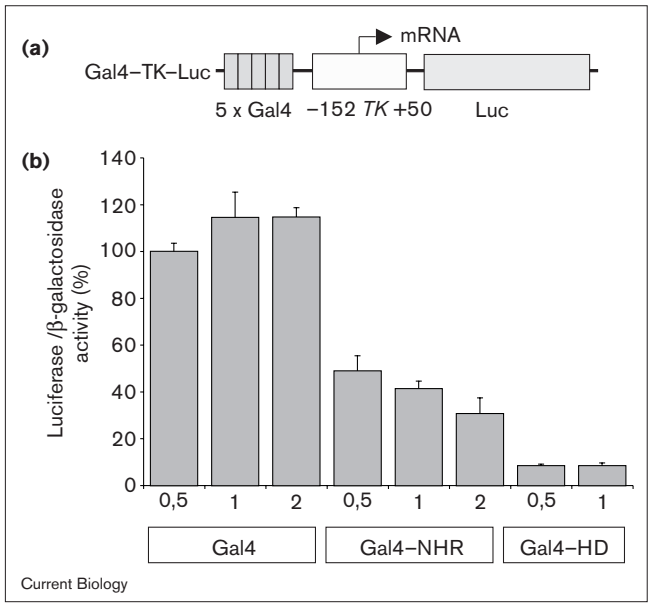


Incorporation of GFP–histone fusions into chromatin. **(a–e)** Absence of diffusion of GFP–H2A and GFP–macroH2A but not of GFP–NHR in transfected living human fibroblast nuclei. Human primary fibroblasts were transfected and, 24 h post-transfection, the GFP fluorescence of the living cells was measured (0 sec). The boxed region (0 sec, left panel) is shown magnified in the five panels on the right. The zone indicated in the 0 sec, right panel was then bleached according to the FRAP technique and the fluorescence recovery recorded at the indicated times after bleaching. Arrowheads indicate the irradiated regions in each panel. **(f–h)** GFP–histone fusion proteins were incorporated into the Barr body chromatin. Confocal sections including the GFP-labeled Barr body (arrowheads) were chosen and irradiated as above.

between these possibilities, GFP fusions with macroH2A (GFP–macroH2A), the H2A-like (amino acids 1–120; GFP–H2A-like) and non-histone regions of macroH2A (amino acids 121–371; GFP–NHR), and to the regular histone H2A (GFP–H2A), were expressed in normal female fibroblasts (Figure 2). The GFP–macroH2A fusion proteins preferentially labeled the Barr body (Figure 2b). This pattern of labeling was strictly dependent on the presence of the histone H2A-like region, as a GFP–NHR was homogeneously distributed in the nucleus and did not label the Barr body (Figure 2d). In agreement with these immunolocalization results, the Barr body was also preferentially labeled with GFP–H2A, clearly showing the presence of a higher density of nucleosomes in this structure compared with the rest of the nucleus (Figure 2e).

To show that the GFP–histone fusions were effectively incorporated in chromatin, fluorescence recovery after photobleaching (FRAP) [5] experiments were performed. Fibroblasts were transfected to compare the diffusion rates of different GFP fusion proteins in living cells. In these experiments, histones incorporated into nucleosomes were expected to show a lower rate of diffusion than free proteins. GFP fluorescence was locally bleached with a laser beam and the rate of fluorescence recovery in the bleached regions measured. During the period of time considered, no diffusion of GFP–macroH2A or GFP–H2A was observed (Figure 3b,e), whereas the diffusion of GFP–NHR or GFP alone was rapid, with a recovery time of less than 5 seconds: no bleached region could be observed 5 seconds after laser irradiation (Figure 3a,d). This rapid recovery did not happen when fixed cells expressing the GFP–NHR or GFP alone

Figure 4



The non-histone region of macroH2A can repress transcription. **(a)** Schematic representation of the luciferase reporter Gal4–TK–Luc. The five Gal4-binding sites upstream of the thymidine kinase promoter (*TK*) are indicated. **(b)** HeLa cells were transfected with 1 μg of the reporter plasmid together with the indicated amounts of expression vectors encoding Gal4 DNA-binding domain alone (Gal4) or Gal4 DNA-binding domain fused to the non-histone region of macroH2A (Gal4–NHR). Gal4–HD indicates an expression vector encoding the Gal4 DNA-binding domain fused to the histone deacetylase domain of HDAC5, shown to efficiently repress transcription [10]. In all transfections, 100 ng of a cytomegalovirus (CMV) β-galactosidase reporter plasmid was also used for normalization purposes; 24 h post-transfection, luciferase activity was measured and normalized with respect to that of β-galactosidase (± SD).

were bleached (data not shown), confirming that the rapid recovery of fluorescence in the living cells was due to the high rate of diffusion of these molecules. In the above experiments, the general association of GFP–histone fusions with chromatin was shown by bleaching random spots. We then checked that our conclusions were also true for the Barr body. In cells transfected with GFP–histone fusions, confocal sections including the Barr body were chosen and this territory was bleached as above. We found that, as in the rest of the nucleus, the expressed GFP–histone fusions were tightly associated with chromatin in this structure (Figure 3f–h). Finally, the ability of the GFP–macroH2A to integrate into chromatin was assessed by analyzing the mitotic chromosomes of transfected cells and, as expected, the incorporation of GFP–macroH2A into chromosomes was strictly dependent on the presence of the histone region of the protein (data not shown).

The specificity of the association of macroH2A with the inactive X chromosome therefore needs to be reconsidered. Our GFP–histone expression approach and the immunodetection experiments demonstrated a higher density of histones, and thus nucleosomes, in the inactive X chromosome. In a previous study, a comparison of the volumes of inactive and active X chromosome territories in interphase female nuclei by FISH showed that the volumes of the two X chromosomes did not significantly differ [6]. Also, a detailed study performed by Verschure and colleagues [7], analyzing chromosome territories, showed the presence of sub-structures in both X chromosome territories. These observations and those reported here suggest a model in which specific regions of the inactive X chromosome, rather than the whole chromosome territory, display a compact chromatin structure. The organization of these sub-structures might also account for our observation of a denser chromatin domains in the inactive X chromosome.

What could the functional role of macroH2A be? The association of macroH2A with specific nucleosomes may participate in creating transcriptionally inactive chromatin regions through the incorporation of silencers via its long non-histone part. To test this hypothesis, we fused the non-histone region of macroH2A to the Gal4 DNA-binding domain, to target this particular domain into the thymidine kinase promoter flanked by five Gal4-binding sites, a classical experiment to show the repressive activity of chromatin-associated proteins such as MeCP2 [8,9]. Figure 4 shows that the presence of the non-histone domain of macroH2A at the proximity of this promoter can efficiently hinder its transcriptional activity. This experiment suggests that the non-histone region of macroH2A is capable of recruiting specific nuclear proteins to control the activity of regions of chromatin containing macroH2A. The non-histone part of macroH2A may therefore constitute a signal that would initiate the assembly of specific complexes, which would in turn create transcriptionally inactive regions.

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