High Mobility Group Protein N5 (HMGN5) and Lamina Associated Polypeptide 2 alpha (LAP2α)
Interact and Reciprocally Affect Their Genome-wide Chromatin Organization*

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*Running title: Interaction and chromatin binding of HMGN5 and LAP2α

#Equal contributions

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CAPSULE.

Background: HMGN5 is a nucleosome binding protein that affects chromatin structure and function and Lap2α is a lamin binding protein that can also bind to DNA.

Results: HMGN5 and LAP2α interact and reciprocally affect each other’s genome-wide distribution.

Conclusion: Nucleosome-binding proteins and lamin-binding proteins interact functionally.

Significance: We report a novel type of link between the chromatin fiber and the nuclear lamin network.

ABSTRACT.

The interactions of nuclear lamins with the chromatin fiber play an important role in regulating nuclear architecture and chromatin function; however, the full spectrum of these interactions is not known. We report that the N-terminal domain of the nucleosome binding protein HMGN5 interacts with the C-terminal domain of the lamin binding protein LAP2α and that these proteins reciprocally alter their interaction with chromatin. Chromatin immunoprecipitation analysis of cells lacking either HMGN5 or LAP2α reveals that loss of either protein affects the genome-wide distribution of the remaining partner. Our study identifies a new functional link between chromatin-binding and lamin-binding proteins.

INTRODUCTION.

The structure and activity of chromatin is regulated by the dynamic binding of numerous nuclear proteins to their nucleosomal targets. Many of these proteins are targeted to specific sites by unique DNA sequence motifs or specific histone modifications. On the other hand, architectural proteins such as the linker histone H1 (1,2) and high mobility group (HMG) proteins (3-5) bind to chromatin without any obvious specificity for DNA sequence or histone modification. The mechanisms regulating their interactions with chromatin are not fully understood. Members of the high mobility group N (HMGN) protein family bind specifically to the nucleosomes (6) throughout the genome (7). However, these interactions are not random; genome-wide analysis revealed that the HMGN1 variant preferentially localizes to regulatory regions such as enhancers and promoters (7). Elucidation of the factors that affect the interaction of HMGNs with chromatin is important since these proteins affect chromatin compaction, the levels of histone modification, and the fidelity of transcription.

HMGN proteins have a disordered primary structure and associate with numerous nuclear proteins (8,9). These associations could conceivably affect the chromatin interactions and function of HMGNs. Here we report that the human HMGN5 variant (10) interacts with the lamina associated polypeptide 2α (LAP2α). LAP2α is a LEM domain protein that interacts with A-type lamins (11). However, unlike most other LEM domain proteins, LAP2α lacks a transmembrane domain and localizes throughout the nucleus where it interacts with the nucleoplasmic pool of A-type lamins (12) and also binds DNA, either directly or through an association with the chromatin-binding protein Barrier to Autointegration Factor (BAF) (13).

Given that both HMGN5 and LAP2α bind to chromatin and because their binding partners could potentially affect these interactions, we tested whether changes in the levels of either HMGN5 or LAP2α affect the nuclear dynamics and chromatin interactions of their partner. By fluorescence recovery after photobleaching (FRAP) and chromatin immunoprecipitation analysis (ChIP), we find that indeed, the proteins mutually affect their interaction with chromatin.

The nuclear lamina is in direct contact with the chromatin fiber, and changes in these contacts or alterations in the nuclear lamin network can affect chromatin structure and function and influence the cellular phenotype (13-15). Our studies reveal a functional connection between nucleosome-binding and lamin-binding proteins and suggest an additional
link between the chromatin fiber and the nuclear lamin network.

**EXPERIMENTAL PROCEDURES**

**Cell lines**—The HEK-293T and HeLa cells were maintained in DMEM containing Glutamax and supplemented with 10% FBS, 37 °C, 5% CO2, H2O saturated. HeLa cells stably expressing Lap2α-shRNA or control shRNA cells were previously described (16).

**Plasmids and transfection**—The HaloTag-HMGN5 was constructed using the pHTC Halo-Tag CMV (Promega). The pEGFP-HMGN5 and pEGFP- LAP2 plasmids were constructed by subcloning the respective cDNA into pEGFP-N1 vectors. For siRNA-mediated down regulation of HMGN5, HeLa cells were transfected with specific or control siRNA “ON TARGET plus SMART pool” from Dharmacon using Dharmafect 4 transfection reagent.

**Fluorescence recovery after photobleaching (FRAP)**—HeLa cells were transiently transfected, as described above, with Lap2α-Cherry and HMGN5-YFP expressing plasmid and incubated for 24 hour and analyzed as described (17). Each experiment was repeated at least 3 times.

**Antibodies**—Affinity pure rabbit anti hHMGN5, (HPA000511) and anti-Actin (A5316) were from Sigma, anti-Halo from Promega Corporation Company (cat #G928A), anti Lap2α from Milipore, secondary antibodies from Jackson Immunoresearch, HRP-conjugated for Western blots from Millipore.

**HaloTag and GST pull-down**—For HaloTag pull-down, cells expressing HaloTag fusion proteins were lysed in Mammalian Lysis Buffer (Promega) and processed according to manufacturer’s instructions. Protein samples were analyzed by either mass spectroscopy or western blot. 35S-LAP2α recombinant protein were prepared with TnT T7 Quick Coupled Transcription/Translation System (Promega), according to manufacturer’s instructions. For the in vitro GST pull-down assays, GST-fusion HMGN5 proteins were incubated with 35S-LAP2α recombinant protein for 1h at room temperature and then adsorbed on glutathione Sepharose beads (Amersham) for 2 h at 4 °C, washed three times with PBS, resolved by 10% SDS–PAGE, and autoradiographed.

**ChIP/Re-ChIP and ChIP-seq assays**—HeLa cells were grown on a 150-mm culture dish to approximate 80% confluence fixed with 1% formaldehyde (Thermo scientific) for 10 minutes at room temperature. Cross-linked chromatin was then sheared by sonication to 200–700 base pairs (bp) fragments with misonix sonicator 3000. First immunoprecipitation was performed with Dynabeads Protein G Immunoprecipitation Kit (life technologies) using Lap2α antibody. Immunoprecipitated DNA-protein complex was eluted in 100 µl elution buffer. 10µl of chromatin samples were used as control for the first ChIP reaction. The remaining chromatin was diluted to 2 ml with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.0, 167 mM NaCl, and protease inhibitor freshly added). Each 1 ml aliquot was incubated with either HMGN5 antibody or normal rabbit IgG. DNA was extracted with MinElute purification Kit (Qiagen) after reverse-crosslinking and subjected to PCR analysis. Primers used in PCR are available upon request. ChIP-seq library was prepared following the manufacturer’s instructions (Illumina Part# 11257047 Rev.A).

**Preliminary ChIP-seq analysis**—Reads were aligned to the hg19 (Feb. 2009) build of the human genome using bowtie (18) with parameters: -n 2 –a –m 10 –strata --best. Score profiles for visualization on the UCSC Genome Browser (19) were generated as described previously (20).

**Peak finding**—Regions of enrichment (“peaks”) for each ChIP-seq library were identified using SICER (21) with the parameters: redundancy threshold = 1, window = 200, fragment size = 200, gap size= 200, FDR = 0.01.

**Scatter plots**—Scatter plots for HMNG5 and LAP2α (Fig. 3 A, B) were constructed by first obtaining the union set of peaks for HMGN5 and LAP2alpha. Read counts for both libraries were summed for each peak in the union set and the log of the counts on each peak was plotted. The union peak set was classified into ‘Promoter’ peaks that were within 1kb of an annotated UCSC gene TSS and ‘Distal’ peaks otherwise. Promoter peaks were further classified as corresponding to expressed (‘Exp’)
or non-expressed (‘Non-exp’) based on FPKM values of the corresponding genes (see RNA-seq analysis). Expressed genes were determined as detected (FPKM > 0) is two replicate experiments. A more conservative threshold to determine detected genes (FPKM > 1) was also tried but this did not alter the results.

Alignment profiles—Alignment profiles (Fig. 3 C, D) were constructed as described previously (20). FASTQ files for HeLa cell RNA-seq data were downloaded from the ENCODE project at UCSC. FPKM values were obtained with TopHat (22) and Cufflinks (23).

MNase digestion assay—MNase digestion assay was performed using EZ Nucleosomal DNA Prep Kit (ZYMO RESEARCH) according to manufacturer’s instruction.

RESULTS AND DISCUSSION

Specific interaction between HMGN5 and LAP2α—To identify potential HMGN5 binding partners, we stably transfected HEK-293T cells with vectors expressing either HMGN5-Halo or unconjugated Halo Tag, and used HaloLink affinity chromatography of extracts from these cells to search for proteins that specifically interacted with Halo-HMGN5 (Fig.1A). Mass spectrometry analysis of the affinity purified fractions on SDS-PAGE gradient gels revealed two bands that specifically interacted with Halo-HMGN5 (Fig.1A). Mass spectrometry analysis of the bands identified several putative HMGN5-interacting proteins including the lamin binding protein LAP2α.

Co-immunoprecipitation experiments with extracts from cells co-expressing LAP2α-myc and either Halo-HMGN5 or unconjugated Halo, verified that HMGN5 and Lap2α interact in living cells (Fig.1B). Furthermore, co-immunoprecipitation with extracts from cells coexpressing Halo-HMGN5 and either LAP2α-GFP, its C-terminal deletion mutant LAP2αΔ188-693-GFP, or its N-terminal deletion mutant LAP2αΔ1-187-GFP, indicated that HMGN5 interacts with the C-terminal region of LAP2α (Fig. 1C). In vitro co-immunoprecipitation experiments of 35S-labelled LAP2α, or its C-terminal and N-terminal deletion mutants, with purified GST labeled HMGN5, HMGN5Δ67-282 or HMGN5Δ1-66 revealed that the two proteins interact directly, and that this interaction involves the N-terminal region of HMGN5 and the C-terminal region of LAP2α (Fig. 1D).

LAP2α affects the nuclear dynamics of HMGN5—Given that both HMGN5 and LAP2α are nuclear proteins and interact in living cells, we examined their organization in the nuclei of cells expressing both LAP2α-Cherry and HMGN5-YFP. We find that both LAP2α and HMGN5 are distributed throughout the nucleus, but HMGN5 appears to be enriched in heterochromatin, as previously reported (10). The merged images and the corresponding localization profile suggest extensive colocalization throughout the nucleus; however the relative amounts of HMGN5 to LAP2α are higher in the condensed heterochromatin regions and lower in regions depleted of condensed chromatin, which appear to be enriched in LAP2α (Fig. 2A,B).

The intranuclear organization of many chromatin binding proteins, including all the members of the HMGN protein family, is highly dynamic; the proteins continuously roam throughout the nucleus and interact transiently with their binding sites, often within a protein network in which the binding of the various members to their specific sites is interdependent (1,24,25). Given the extensive colocalization of HMGN5 and LAP2α throughout the nucleus, and in view of our finding that the proteins interact in living cells, we used FRAP to examine whether the proteins mutually affect their intranuclear dynamic properties. To this end, we used HeLa cells depleted of LAP2α (16), or cells depleted of HMGN5 by siRNA treatment. Immunofluorescence analysis indicates that down regulation of one of the protein did not affect the intranuclear distribution of the remaining partner (not shown). Western analysis of these cells verified that the level of each protein was significantly down regulated (Fig. 2C).

We find that downregulation of LAP2α affected the intranuclear dynamics of HMGN5. The time required to recover 50% of the HMGN5 pre-bleach fluorescence intensity was 4 seconds in control HeLa cells, but over 8 seconds in HeLa cells lacking LAP2α; a two
fold increase in fluorescence recovery time (Fig. 2D), indicating that the mobility of HMGN5 was significantly reduced in the absence of LAP2α. Conceivably, Lap2α may affect the dissociation rate of HMGN5 from chromatin. The fluorescence recovery time of LAP2α is extremely short and was not affected by loss of HMGN5 (Fig. 2F). However, upregulation of HMGN5 levels increased the time required to reach 80% of the LAP2α pre-bleach fluorescence intensity from 2 seconds in wild type HeLa cells, to 3 seconds in cells overexpressing HMGN5 (Fig. 2E). The difference is not due to experimental manipulation since transfection with control, empty vector did not affect the FRAP recovery curves of LAP2α. Thus, alteration in the cellular levels of LAP2α affects the intranuclear dynamics of HMGN5 while alteration in the levels of HMGN5 has a slight effect on the mobility of LAP2α.

Reciprocal effects of HMGN5 and LAP2α in their genome-wide distribution—FRAP kinetics provide information on the apparent intranuclear mobility of the fluorescent proteins which in part is dependent on their residence time on an immobile surface such as chromatin. For HMGN proteins, it has been directly demonstrated that point mutations that abolish the specific binding of HMGN to nucleosomes increase their intranuclear mobility (26). LAP2α interacts with nuclear lamins and with DNA, albeit with a very short residence time as indicated by our FRAP analysis. Given that both LAP2α and HMGN5 bind to chromatin, and in view of our findings that the proteins interact and mutually affect their intranuclear mobility, we used ChIP-seq (27) analysis to examine directly their genome-wide organization and to test whether the loss of one protein affects the chromatin binding of its partner.

In the chromatin of control HeLa cells, we identified a total of 63,088 HMGN5 binding sites and a total of 22,400 Lap2α binding sites. Of the HMGN5 sites, 7,772 (12%) overlapped with LAP2α sites, while 55,316 (88%) did not. Of the LAP2α binding sites, 7,772 (35%) overlapped, while 14,628 (65%) did not with HMGN5 sites (Fig. 3E). ChIP/Re-ChIP analysis of 3 different loci verified the concurrent occupancy by both LAP2α and HMGN5 at specific sites (Fig. 3G). Genome wide, 23% of the overlapping sites, 16% of the HMGN5-specific sites, and 6% of the LAP2α-specific sites, localized to proximal promoters with no difference between active and inactive promoters. In the overlapping sites, genomic sites where LAP2α binds strongly also demonstrate strong binding of HMGN5, with a stronger correspondence at peaks in promoter regions, regardless of expression of the corresponding gene (Figure 3A,B).

Downregulation of LAP2α decreased the binding of HMGN5 (Fig. 3C), while loss HMGN5 only slightly reduces the binding of LAP2α (Fig. 3D). The reciprocal effects of HMGN5 and LAP2α on their genome-wide distribution are summarized in Figure 3E,F. Downregulation of either HMGN5 or LAP2α levels led to significant alterations in the interaction of both HMGN5 and LAP2α with chromatin (Fig. 3F). Downregulation of LAP2α led to loss of HMGN5 from 77% of the HMGN5 specific sites (class IV in Fig. 3F) and from 58% of the HMGN5 sites that overlapped with LAP2α (class II). In addition, loss of LAP2α created 15,583 new HMGN5 binding sites, and of these, 14,008 sites were novel, i.e. not previously bound by either HMGN5 or LAP2α, and 1,575 sites were loci that previously contained only LAP2α (Class V and class VI sites, Fig. 3F, left).

Likewise, downregulation of HMGN5 led to significant changes in the genome-wide organization of LAP2α. Downregulation of HMGN5 resulted in the loss of 90% of sites occupied only by LAP2α and 86% of sites commonly occupied by both proteins, as well as the creation of 15,483 new LAP2α binding sites (Class V and VI sites, Fig. 3F right). The total number of LAP2α binding sites in cells lacking HMGN5 was 18,610 i.e. 83% of the number of sites present in wild type cells. Thus, loss of HMGN5 changed mostly the location, rather than the number of LAP2α binding sites.

Previous studies indicated that human HMGN5 affects chromatin structure (10). Given the effect of LAP2α on the FRAP kinetics and the genome-wide organization of HMGN5, we tested whether loss of LAP2α induces major changes in chromatin organization. Comparison of the micrococcal nuclease digestion patterns of
nuclei isolated from wild type and LAP2α-deficient HeLa cells revealed that neither the kinetics of digestion, nor the nucleosomal repeat is affected by loss of LAP2α (Fig. 4). Thus, the altered organization of HMGN5 in LAP2α-deficient cells is not due to major changes in the nucleosomal organization of the chromatin fiber. Nevertheless, previous studies demonstrated that loss of just one LEM protein or HMGN variant can affect chromatin function. Our studies indicate that loss of either HMGN5 or LAP2α protein affect the dynamic binding of its protein partner to chromatin

Increasing evidence indicates that interactions between the nuclear lamin network and chromatin play an important role in nuclear architecture and gene expression. The results presented here reveal an additional link between the chromatin fiber and the lamin network formed by the interaction between a nucleosome-binding protein and a lamin-binding protein.

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LEGENDS TO FIGURES

Figure 1. HMGN5 and LAP2α interact in vivo and in vitro. A, silver-stained SDS-PAGE gel of proteins recovered from HaloLink resin. Specific bands are marked as 1,2. B, immunoprecipitation of LAP2α-myc by Halo-HMGN5 from HeLa cell extracts. C, HMGN5 interacts with the C-terminal of LAP2α. Shown are westerns of input and immunoprecipitated proteins from HeLa cells expressing Halo-HMGN5 and LAP2α-GFP constructs. D, GST-pull down assay indicates that the N-terminal of HMGN5 interacts with C-terminal of LAP2α. 35S-labelled LAP2α, or its deletion mutants, were incubated with purified GST-HMGN5 or its deletion mutants, the complex was purified on glutathione-Sepharose, fractionated by SDS-PAGE, and visualized by autoradiography. The scheme visualizes that the C-terminal region of LAP2α interacts with the N-terminal region of HMGN5.

Figure 2. Mobility of HMGN5 and LAP2α in wild type and knock-out cells. A, localization of HMGN5 and LAP2α in HeLa cells. Arrow points to euchromatic regions. B, localization profiles along the paths depicted by the lines drawn in (A). C, Western analysis reveals efficient down-regulation of either HMGN5 or LAPα levels in HeLa cells. D-F, FRAP recovery curves. The protein analyzed and the types of cells used are indicated on top of each panel.

Figure 3. Reciprocal effects of HMGN5 and LAP2α on their genome-wide distribution. A-B, LAP2α and HMGN5 preferentially co-localize at promoters. C, loss of LAP2α reduces the binding of HMGN5 across the genome. D, Loss of HMGN5 slightly reduces the binding of LAP2α across the genome. E, chromatin binding sites are indicated as: Red: HMGN5-specific binding sites; Green: LAP2α - specific binding sites; Yellow: binding sites for both HMGN5 and LAP2α; Black: chromosomal regions with no HMGN5 or LAP2α binding. F, genome-wide distribution of HMGN5 before and after LAP2α knockdown (left panel). Genome-wide distribution of LAP2α before and after HMGN5 knockdown (right panel), change of protein bindings is color-coded as indicted in (E). The % of sites retained or lost upon deletion of either HMGN5 or Lap2α is indicated for each class of binding sites. G, re-ChIP validation of genomic regions associated with both LAP2α and HMGN5. Chromatin was first immunoprecipitated with LAP2α antibody, followed by a second round of ChIP with HMGN5 antibody. The protein profile at the genomic location analysed is indicated. The positions amplified for Q-PCR analysis are indicated by arrows.

Figure 4. Loss of LAP2 alpha does not change overall chromatin structure. Shown is a gel depicting the kinetics of micrococcal nuclease digestion in wild type and LAP2 alpha deficient HeLa cells. 1.5×10⁶ nuclei were treated with 0.01-1 units of micrococcal nuclease in 100 μl digestion buffer for 10 minutes at room temperature. The purified nucleosomal DNA was fractionated on 2% agarose gel and quantified with ImageQuant software. The scans of corresponding lanes from the two different digests indicate that neither the kinetics of digestion nor the nucleosomal repeat is affected by loss of LAP2 alpha.
Zhang et al. Figure 1.
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Zhang et al Figure 4.
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