The linker histone H1.2 is a novel component of the nucleolar organizer regions

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Running title: Histone H1.2 recruitment to NORs

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The nucleoli accumulate rRNA genes (rDNA) and are the sites of rRNA synthesis and rRNA assembly into ribosomes. During mitosis, nucleoli dissociate but nucleolar remnants remain on the rDNA loci, forming distinct nucleolar organizer regions (NORs). Little is known about the composition and structure of NORs, but upstream binding factor (UBF) has been established as its master organizer. In this study, we sought to establish new proteins in NORs. Using UBF-Sepharose to isolate UBF-binding proteins, we identified histone H1.2 as a candidate partner, but were puzzled by this observation given that UBF is known to be located predominantly in nucleoli whereas H1.2 distributed broadly among the chromatins in interphase nuclei. We then examined cells undergoing mitosis, and saw that both H1.2 and UBF were recruited into NORs in this state, reconciling the results of our UBF pulldowns. Inhibiting rRNA synthesis in interphase nuclei also induced NOR-like structures containing both UBF and H1.2. When chromosomes were isolated and spread on coverslips, NORs appeared separated from the chromosomes containing both UBF and H1.2. After chromosomes were fragmented by homogenization, intact NORs remained visible. Results collectively suggest NORs are independent structures and the linker histone H1.2 is a novel component of this structure.

Nucleoli are distinct nuclear bodies in which rRNA genes (rDNA) are congregated and rRNA is synthesized and assembled into ribosomes (1-3). At interphase, nucleoli occupy significant nuclear domains around the rDNA loci. At mitosis, nucleoli disintegrate but remnants remain on rDNA, forming the nucleolar organizer regions (NORs) (4,5). The structure of interphase nucleoli was proposed largely based on transmission electron micrographs with an overall tripartite organization (3,6). However, the composition and structure of NORs remain poorly delineated (7).

Based on the tripartite model, an interphase nucleolus is composed of one or more fibrillar centres (FC) each being surrounded by a dense fibrillar component (DFC) and these FC/DFC units are embedded in a greater granular component (GC) (8-10). These nucleoli are encircled by a dense layer of perinucleolar heterochromatin but chromatins are otherwise scarce in the nucleolar lumen (11,12). In live cells, nucleolar proteins can be highly dynamic and mobile (13). In fact, liquid-like properties have been ascribed to nucleoli and other nuclear domains (14,15). Nonetheless, nucleoli still display substantial structural independence and can be isolated from homogenized nuclei (12,16,17). The structural independence of NORs has not been assessed.

The tripartite nucleolar structural model aligns well with the known function of nucleoli in ribosome generation. rDNA and elements of the rRNA transcription machinery are located in the innermost FC regions (3,10). rRNA transcription takes place at the FC/DFC interface where newly synthesized pre-rRNA accumulates (18,19). Each rRNA gene can be simultaneously transcribed in tandem by multiple RNA polymerase I (Pol I), giving rise to tandem rRNA transcripts that stem from each rDNA locus like a ‘Christmas tree’ (20,21). rRNA is
processed in the DFC region and incorporated into ribosomes in the GC region (3,10).

During mitosis when rRNA transcription arrests, nucleoli disintegrate but nucleolar remnants remain associated with the rDNA loci to form NORs (7,22). Some elements of the nucleolar FC domains are recruited into NORs but GC and DFC elements disperse at this stage (23,24). Like the chromatids, NORs also divide equivalently into daughter nuclei and then re-emerge as nucleoli (22). The rDNA loci reside on acrocentric chromosomes and NORs form adjacent to these rDNA loci which, unlike the rest of the chromosomal regions, assume distinctly open configurations (25,26). These nucleolar remnants precipitate silver nitrite and are therefore also known as AgNORs (27). The detailed NOR structure and composition, however, are not understood and the rDNA loci remain incompletely sequenced (7).

Upstream binding factor (UBF), a Pol I-associated transcription factor, is a nucleolar protein that appears essential to both NOR and nucleolus formation around the rDNA loci. Each active rRNA gene contains a core promoter, which is recognized by selective factor 1 (SL1), and it also contains an upstream enhancer element which is bound by UBF. These jointly recruit Pol I to initiate rRNA transcription (28).

In *Xenopus*, UBF binds not only to the rDNA enhancer but also broadly to other sites in the rDNA locus (29,30). Ectopic introduction of these tandemly engineered *Xenopus* UBF-binding elements into human HT1080 cells demonstrated recruitment of human UBF and Pol I transcription elements to form pseudo-NORs at mitosis and induce nucleolar FC-like structures at interphase (31). When the ectopic DNA construct was completed with rRNA-coding sequences, these engineered neo-rDNA loci were able to develop into nucleoli in interphase nuclei, producing rRNA and ribosomes (32). This demonstrates the instrumental roles of UBF in the structure and functions of NORs and the nucleoli.

We argued that, by identifying new UBF-binding proteins in the nuclei, novel insights could be obtained into the structure and functions of the nucleoli and NORs. Using UBF-Sepharose, we have identified the linker histone H1.2 as a prominent UBF-binding protein and it was shown to follow UBF into NORs. These NORs containing UBF and H1.2 were structurally separable from chromosomes.

Results

**Generation of nuclear extract**

To identify UBF-binding proteins, we first prepared a soluble extract from isolated nuclei. Nuclei rather than nucleoli were used because UBF also binds to selected genes outside the nucleoli and regulates RNA polymerase II (Pol II)-mediated gene expression (33-35). The nuclei were first extracted with Triton X-100 to deplete the nuclear envelope and these nuclei, known as TxN, mostly remained intact and oval and retained the nuclear protein profile (data not shown, Figure 1A). Proteins were then test-extracted from TxN at increasing NaCl concentrations (100-500 mM). Protein extraction plateaued at 400-500 mM NaCl (Figure 1A). The otherwise particulate TxN burst into one colloidal gel at 500 mM NaCl (data not shown), suggestive of dissociation of the nuclear scaffold from the chromatin. The nuclear extracts lacked significant core histones which ruled out significant chromatin contamination (Figure 1A). For affinity pulldowns, nuclear extract was routinely extracted from TxN at 500 mM NaCl, known as TxNE. Briefly, nuclei were isolated after centrifugation through 2.2 M sucrose and extracted with Triton X-100 to generate TxN (Figure 1A). TxNE is obtained by extracting TxN at 500 mM NaCl.

The inclusiveness of TxNE as a nuclear extract was surveyed by detecting representative nucleolar and nuclear proteins (Figure 1B-1K). The nucleolar proteins nucleophosmin-1 (NPM1), nucleolin, UBF and fibrillarin were all detectable from the 300-mM NaCl extract (Figure 1B-1E). The intimately chromatin-associated proteins, heterochromatin protein 1γ (HP1γ), Pol II, lamin A/C, and topoisomerase IIα (TopoIIα), were also extracted (Figure 1F-1I). In contrast, centromere protein A (CenP-A) and the nuclear lamina protein lamin B1 (LB1) were absent (Figure 1J and 1K). Overall, TxNE is highly inclusive of nuclear proteins without significant chromatin contamination.

To view changes in nuclear chromatin organization upon NaCl extraction, TxN were first adhered to coverslips and then exposed to 100-500 mM of NaCl. TxN became increasingly swollen at rising NaCl concentrations (data not shown). At 500 mM NaCl, TxN burst into extended and often parallel chromatin fibers (Figure 1L). The otherwise continuous nuclear lamina, as identified by LB1, fractured and scattered among the chromatin fibers (Figure 1L).
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Histone H1.2 is a novel UBF-binding protein

UBF-Sepharose was prepared from recombinant UBF and used in affinity chromatography with TxNE. Proteins were eluted from UBF-Sepharose using 500 mM NaCl which showed heterogeneity in size (Figure 2A). By LC-MS/MS (Supplemental Table S1), a group of four high molecular weight proteins were all identified as nucleolin (Figure 2A). A 36-kDa band was identified as NPM1. A 28-kDa region was abundant with a H1 histone variant H1.2 (Figure 2A). Being nucleolar proteins, association of nucleolin and NPM1 to UBF-Sepharose was not surprising. However, H1.2 was not known to bind to UBF or to localize to the nucleoli.

H1.2-UBF interaction was further examined using H1.2-Sepharose and, as a control, Tris-Sepharose. After TxNE was applied on Tris-Sepharose, UBF remained abundant in the flow through fraction and was not subsequently eluted from the resins (Figure 2B). With H1.2-Sepharose, UBF was depleted in the flow through fraction and subsequently eluted from the resins, suggesting UBF binding to H1.2. High mobility group box 1 (HMGB1) is structurally similar to UBF (36), but it showed no binding to Tris- or H1.2-Sepharose (Figure 2B). This shows specific H1.2 binding to UBF.

Whether H1.2 interacts directly with UBF was evaluated with recombinant H1.2 and UBF proteins. When purified H1.2 was applied on UBF-Sepharose, it was completely depleted in the flow through fraction and was subsequently eluted from the resins, showing direct H1.2-UBF interaction (Figure 2C). Likewise, purified UBF also bound to H1.2-Sepharose (Figure 2D). This H1.2-UBF interaction was apparently disrupted at 500 mM NaCl, a condition at which bound UBF and H1.2 were eluted from the resins and TxNE was also eluted from the chromatin network (Figure 1).

H1.2 contains three clearly demarcated domains: a central globular domain (GD) of ~80 amino acid residues, a short N-terminal domain (NTD) and a long C-terminal domain (CTD) of ~100 residues. We then examined which of the three H1.2 domains bound to UBF. To this end, these H1.2 mutants were generated by deleting its NTD, CTD or both. As shown in Figure 2E, after the short NTD was deleted, H1.2 still bound to UBF but binding to UBF was abolished when CTD was deleted from H1.2. The H1.2 GD alone showed no binding to UBF. These suggest H1.2 binds to UBF through its CTD, which is more diversified than GD among the H1 variants (37). It is known that the H1 bind to nucleosomes through the GD while the H1 CTD bind more permissively to linker DNA, RNA and potentially protein partners (38).

Disparate UBF and H1.2 distribution in interphase nuclei but co-localization at mitosis

Chromatins are abundant in the nucleoplasm but scarce in the nucleoli and it is counter-intuitive that H1.2, being a known chromatin-binding protein, also binds to UBF which is a nucleolar protein (11). Staining of UBF and H1.2 in HeLa cells indeed revealed broad H1.2 distribution in the nucleoplasm but UBF was concentrated in the chromatin-poor nucleoli (Figure 3). UBF detection in the nucleoplasm was overall sparse (Figure 3). Based on this largely disparate H1.2 and UBF distribution patterns, UBF-H1.2 interaction can only be expected at limited, if any, nucleoplasmic foci.

Examining mitotic cells, however, revealed condensation of both H1.2 and UBF in a number of granular NORs (Figure 3). At sequential stages of mitosis, UBF and H1.2 exhibited distinct kinetics in NOR recruitments. At prometaphase, when all nuclear UBF apparently condensed in nine early NOR granules, only a fraction of H1.2 was found in these mitotic structures. Subsequently at metaphase, however, H1.2 was only detectable in NORs (Figure 3).

During anaphase, NORs appeared to divide equivalently like the chromatids based on the intensity of UBF in the divided NORs (Figure 3), as previously reported (22). H1.2 was also equivalently divided. At telophase, the chromatins began to unwind and UBF also began to reorganize into nucleoli. H1.2 rapidly exited the nucleolar regions and became detectable among the unwinding chromatin network (Figure 3). Mitotic cells develop NORs that contain different amounts of UBF. H1.2 appears to be recruited into these NORs in proportion with UBF. Since UBF is a housekeeping protein in NORs (31,32), H1.2 could be recruited through its interaction with UBF.

H1.2 recruitment to NORs is a selective event
In order to gauge whether other nuclear proteins besides H1.2 are also recruited into mitotic NORs, mitotic cells were stained with a list of antibodies including those used in the Western blotting experiment shown in Figure 1A, but none stained the NORs. For example, NPM1 and fibrillarin both localized to interphase nucleoli, but they dispersed from NORs during mitosis instead of being condensed into these structures (Figure 4; data not shown). The human H1 histone family consists of 11 independently coded variants among which six (H1.1-H1.5 and H1.x) are somatically expressed (Supplemental Figure S1) (37). When H1.1, H1.3, H1.4, H1.5 and H1.x were immuno-stained, none were detected in NORs (Supplemental Figure S2), further demonstrating that H1.2 recruitment into NORs is a selective event. Interestingly, H1.x was concentrated in the interphase nucleoli (Supplemental Figure S2). This was also observed in a previous study (39). However, it dispersed at mitosis like fibrillarin (Figure 4). H1.0 and H1.4 were also significantly detected outside the nuclei.

To examine whether other H1 variants also interact with UBF, recombinant H1.1, H1.3, H1.4, H1.5 and H1.x were similarly generated. In a pulldown study with UBF-Sepharose, these purified H1 variants were incubated with UBF-Sepharose resins for 2 hr which were then washed and eluted at 1 M NaCl. H1.2 was markedly depleted in the supernatant and subsequently eluted from the resin (Figur 2F). As a control, it was not eluted from Tris-Sepharose. Among the other purified H1 variants, only H1.3 was eluted from the UBF-Sepharose resins (Figure 2F). H1.1, H1.4, H1.5 and H1.x were not detectable in the elution. H1.3 is more closely related to H1.2 than the other H1 variants (Supplemental Figure S1). However, its expression in HeLa cells is too low to draw conclusions (Supplemental Figure S2).

Pol I inhibition causes UBF and H1.2 recruitment into NOR-like structures in interphase nuclei

In interphase cells, NOR-like granules can also be induced when Pol I is inhibited with low concentrations of actinomycin D (ActD) (40,41). When HeLa cells were treated for 2 hr with ActD (40 ng/ml), UBF was shown to condense into NOR-like granules of different sizes (Figure 5). H1.2 was proportionally recruited into all these structures, mirroring UBF and H1.2 recruitment into mitotic NORs (Figure 3). It was also noted that only a fraction of total nuclear H1.2 and UBF condensed in the ActD-induced NORs with the rest of each protein being broadly distributed among nucleoplasmic chromatin (Figure 5). These two distinct pools of UBF and H1.2 may vary in the extents of their chromatin and other nuclear associations.

The nucleolar protein nucleolin was similarly stained in ActD-treated cells. Instead of being condensed into NORs, it dispersed from its original nucleolar locations and distributed broadly in the nuclei (Figure 5). This shows that H1.2 recruitment to the UBF-demarcated NOR-like structures is selective for a distinct pool of H1.2. In both mitotic and ActD-induced recruitments, the arrest of rRNA transcription appeared to be a shared cause.

The cellular H1.2 levels were similar at interphase and metaphase

The detection of two pools of H1.2 in the ActD-treated interphase nuclei raised different interpretations with respect to the NOR-associated H1.2: 1) Most chromatin-associated H1.2 was degraded in mitotic cells and therefore the NOR-associated H1.2 only represented a fraction of total H1.2 and 2) All cellular H1.2 was recruited into NORs. Considering the key roles of H1 histone in general chromatin compaction, neither of these are strong possibilities (42). Nonetheless, we measured total cellular H1.2 following Colcemid-induced enrichment of metaphase cells. In these cultures, most HeLa cells are synchronized to metaphase after 15 hr. Cells were treated with Colcemid for up to 24 hr but harvested at 3-hr intervals. Total cellular H1.2 was determined by Western blotting, using UBF, topoisomerase IIα (TopoIIα) and LB1 as controls.

Total cellular UBF level showed steady increase from 9 hr and appeared to plateau by 15 hr (Figure 6A). After 24 hr, Colcemid was removed from the culture and, within 6 hr, total cellular UBF decreased to the basal level and UBF fragments were noticed (Figure 6A). The cellular level of TopoIIα, which is a major scaffold protein in chromatin compaction into chromosomes (43), largely mirrored that of UBF (Figure 6A). In contrast, the total cellular levels of H1.2 and LB1 appeared unchanged throughout the Colcemid treatment. This ruled out significant H1.2 degradation during mitosis (Figure 6A), suggesting that either all cellular H1.2 followed UBF into NORs at mitosis or, as
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a third possibility, the chromatin-associated H1.2 is shielded from antibody detection in the compact chromosomes.

**NORs are separable from chromosomes**

UBF and the rDNA loci are perceived to be the backbones of NORs (31,32). The extent of NOR structural independence relative to the chromosomes remain unclear. With the Colcemid-induced metaphase cells, we examined the physical relationship between NORs and the chromosomes by isolating chromosome assemblies from these cells. Unlike interphase chromatin, the chromosomes are fragile during homogenization. However, many intact chromosome assemblies could be generated with mild homogenization at 18-µm clearance. Some chromosome assemblies spread apart on coverslips, revealing significant gaps among chromosomes and also physical relationships between NORs and the chromosomes (Figure 6B). NORs remained intact but showed clear separation from the chromosomes, containing both UBF and H1.2 (Figure 6C and 6D). They appeared loosely tethered to, rather than being integral parts of, the chromosomes (Figure 6E and 6F). H1.2 remained in complete co-localization with UBF in these NORs (Figure 6G). An additional observation from these spread chromosomes was the presence of H1.2 inside the chromosomes as well as the NORs (Figure 6C and 6F), in contrast to the lack of H1.2 detection in endogenous chromosomes (Figure 3).

**Endogenous chromosomes contain compacted H1.2**

To examine the possibility that endogenous chromosomes compact H1.2 from antibody detection, cells were treated with DNase after fixation and permeabilization. As shown in Figure 7, a 30-min DNase treatment of the cells diminished chromatin in the endogenous chromosomes but NORs remained intact containing both UBF and H1.2. Interestingly, H1.2 also became detectable among the residual chromosomes. Since NORs were still intact and abundant with H1.2, the newly revealed chromosomal H1.2 must have been otherwise compacted by chromatin from antibody detection.

**NORs can be detached from chromosomes**

We also homogenized the Colcemid-enriched metaphase cells under more stringent conditions (10 µm clearance) which fragmented most chromosomes (Figure 8A). In these homogenates, many particles were stained positive for UBF (Figure 8B). These particles were also strongly stained for H1.2 (Figure 8C). Many of these particles lacked major chromatin moieties (Figure 8D-8F). These particles exhibited size heterogeneity like the endogenous chromosome-embedded NORs, suggesting that NORs are structurally robust and remain intact during homogenization which have however broken down the chromosomes (e.g. Figure 8G-8I).

**Conclusions**

NORs are well-known mitotic structures but their structure and functions remain inadequately defined. NORs were known to form around rDNA loci during mitosis through UBF. Our data show that they are structurally independent of the chromosome bodies. The linker H1 histones were known as chromatin-binding proteins that facilitate chromatin compaction. Our data show that, among the H1 variants, H1.2 binds to UBF and, during mitosis, it is recruited to NORs like UBF. Results collectively suggest additional functions for NORs besides organizing the rDNA loci during mitosis and also show selective H1.2 association with this structure. However, the distribution of H1 variants, UBF and other nuclear proteins is probably often determined by more than one binding partner.

**Discussion**

The nucleoli synthesize approximately 50% of all cellular RNA in the form of pre-rRNA (1). Being embedded in the chromatin network, nucleoli can still afford dynamic molecular exchanges and morphological changes (13,14). Nucleoli form around the rDNA loci and, as a master nucleolar organizer, UBF appears to bind persistently to these loci throughout the cell cycle (31,32). While the molecular scaffolds that shape the nucleoli and their mitotic remnants, i.e. NORs, remain limitedly understood, these inevitably involve the two core elements, i.e. rDNA and UBF. In this study, we used UBF-Sepharose to identify novel UBF-binding proteins from the nuclear extract (Figure 1).

Identification of histone H1.2 as a UBF-binding protein was at first contradictory to a subsequent observation that these two proteins
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Histone H1.2 recruitment to NORs distributed disparately in the interphase nuclei. However, this paradox was reconciled at mitosis when the chromatin compact to form chromosomes and the nucleoli mostly dispersed leaving remnants near the rDNA loci to form NORs of different sizes. UBF was condensed in these NORs and H1.2 followed UBF into NORs apparently in proportion with UBF. Both UBF and H1.2 were divided equivalently into daughter nuclei. After division, NORs expanded into nucleoli and H1.2 exited the nucleolar regions demarcated by UBF. These observations illustrate a novel mechanism by which a non-nucleolar protein was selectively recruited into NORs during mitosis.

It is unclear how many other proteins may be similarly or differently recruited into NORs like H1.2 but, among the six H1 variants examined (H1.1-H1.5 and H1.x), the other five were not found in NORs (Supplemental Figure S1 and S2). Although the H1.0 variant was reported to bind to numerous nucleolar proteins (44), it was not concentrated in interphase nucleoli or mitotic NORs (Supplemental Figure S2). While the subcellular localization of H1.3 in HeLa cells was not clearly defined due to its low expression, data overall stress a specific relationship among H1.2, UBF and NORs.

When H1.2 was recruited to NORs, it also diminished in the compacted chromosomes (Figure 3), giving the initial impression that the otherwise chromatin-associated H1.2 in interphase nuclei was depleted from the chromosomes and all transferred into NORs. However, H1.2 was actually present inside chromosomes albeit it was masked from antibody detection. When isolated chromosomes were spread on glass coverslips, H1.2 became detectable in these chromosomes (Figure 5). When endogenous chromosomes were digested with DNase, H1.2 also became detectable in the residual chromosomes (Figure 7). Therefore, it appears that two pools of nuclear H1.2 exist with one binding to UBF and being recruited to NORs and the other binding to the chromatin being compacted inside the chromosomes. H1.2 interaction with UBF appears to require its CTD but this region is also required for H1.2 to interact with DNA and RNA (Supplemental Figure 1). The two pools of H1.2 could result from UBF competition with chromatin and other H1.2-binding partners for the H1.2 CTD. Some undefined post-translational modifications may also influence H1.2 interaction with UBF and the ultimate distribution of H1.2 and other H1 variants (38).

In interphase nuclei, UBF is abundant in the nucleoli but it is also distributed among the nucleoplasmic chromatins (Figure 3). UBF has indeed been reported to associate with selected genes in the nucleoplasm (31-35). As a chromatin-binding protein, the broad distribution of H1.2 in the nucleoplasm is natural. It was its mitotic recruitment into NORs that was not expected although it also became logical considering its newly found affinity for UBF. It is unclear whether this H1.2-UBF interaction persists in interphase nuclei or it is only induced during mitosis. In any case, this interaction was also implied, albeit not validated, in ActD-induced NOR-like structures in interphase nuclei (Figure 5).

This unusual property of histone H1.2 prompted us to ascertain the specificity of the anti-H1.2 antibody and examine its potential cross-reactivity with other antigens. We firstly used this antibody to screen 11 human cell lines, including HeLa cells. By Western blotting, the antibody only reacted with one protein which was equivalent to H1.2 in size (Supplemental Figure S2). We also screened the antibody against six purified H1 variants (H1.1-H1.5 and H1.x) and it only reacted with H1.2 (Supplemental Figure S3). The possibility that the antibody cross-reacted with an epitope which was only induced during mitosis was also ruled out, because no additional proteins reacted with the antibody when metaphase cells were enriched in culture using Colcemid (Figure 6A). Finally, a 2-hr ActD treatment was sufficient to induce NOR-like structures in interphase nuclei and these structures were reacted by the anti-H1.2 antibody. Therefore, the antibody is specific for H1.2.

H1.2 is a non-nucleolar protein and has no known functions in rRNA biosynthesis or ribosome assembly. Its recruitment into mitotic NORs is also in contrast to most indigenous nucleolar proteins that instead exit these structures during mitosis. The implications of this phenomenon are unclear. H1.2 could be passenger molecules that are preserved in NORs during mitosis and are then faithfully transmitted to daughter cells without undergoing the usual dismantling experienced by most other nuclear proteins. During the 1970s-90s, search for nuclear scaffolds that could orient interphase nuclear chromatin identified H1 histone as an adaptor protein between the nuclear scaffolds.
and the chromatin (45). It is possible that the chromatin-associated and UBF-associated pools of H1.2 represent distinct nuclear scaffolds and are preserved in chromosomes and NORs respectively during mitosis for faithful transmission.

The rDNA loci lack classic nucleosomes and are instead organized into distinct conformations by dimeric UBF (46). These were also known as secondary constrictions in pioneer studies that led to modern investigations of the nucleolus (4,7,25,47). These unique conformations may function to recruit and preserve certain nuclear configurations that cannot afford complete scrambling during mitosis. Alternatively, H1.2 may be required to form and maintain this conformation together with UBF and other unidentified and similarly recruited nuclear proteins.

Whether H1.2 indeed represents a preserved scaffold in NORs or is required for NOR formation cannot be concluded based on current data. In mice, this is not expected to be essential to cell survival because H1.2−/− mice exhibit normal phenotype (48). However, inducible shRNA depletion of H1.2 in the human breast cancer cell line T47D caused cell cycle arrest (49). In the human fibrosacoma HT1080 cells, inducible shRNA depletion of UBF similarly caused complete growth arrest (32). Using the human HeLa cells, CRISPR-Cas9 knockdown of H1.2 led to no viable H1.2−/− clones (Data not shown). If H1.2-UBF interaction and H1.2 recruitment to NORs are essential to cell survival, this is probably not compensated by other human H1 variants although this is inconclusive for the most H1.2-related H1.3 which appears to bind to UBF but poorly expressed in HeLa cells.

Besides H1.2 recruitment to NORs, H1.0, H1t and H1.x have been reported to associate with nucleoli in different other contexts. As previously reported and also observed by us, H1.x was concentrated in interphase nucleoli but it dispersed at mitosis like fibrillarin (Supplemental Figure S2, Figure 4) (39). H1t is a testis-specific H1 variant which is also expressed in different cancer cell lines (50). Like H1.x, H1t is also concentrated in interphase nucleoli but it dispersed at mitosis. H1t concentration to nucleoli is likely to involve its binding to the rDNA repeats (50), but this is unclear for H1.x. Using immobilized histone H1.0, Kalashnikova et al pulled down numerous ribosomal and other proteins from the nucleolar extract (44). However, these interactions have apparently not enriched H1.0 to the nucleoli or NORs (Supplemental Figure S2). H1.2 remains the only H1 variant that is known to be recruited to NORs.

Overall, our results demonstrated structural independence of NORs and H1.2 as a novel component of this structure. The different H1 variants exhibit dynamic subcellular distribution which suggests distinct functional mechanisms. While it appears that some H1 variants associate with the nucleoli or nucleolar elements, H1.2 is distinct that it localizes in NORs. The ultimate subcellular localization of each H1 variant is likely to be determined by the multiple binding partners that interact with different regions on these versatile molecules.

**Experimental procedures**

**Cell culture and reagents**

Human cervical adenocarcinoma HeLa cells were cultured in DMEM containing 10% (v/v) HyClone fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine at 37°C and 5% CO2. Colcemid (D1925) and a mouse anti-NPM1 antibody (B0556) were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit antibodies for histone H1.2 (ab17677), lamin B1 (LB1, ab16048), nucleolin (ab22758), fibrillarin (ab5821), centromere protein A (ab13939) and heterochromatin protein 1γ (HP1γ, ab10480) were obtained from Abcam plc (Cambridge, UK). Mouse antibodies for UBF (F9), Lamin A/C (636) and TopoIIα (3F6) were obtained from Santa Cruz Biotech, Inc. (Dallas, TX). Mouse anti-Pol II (CTD4H8) was obtained from Merck Millipore (Darmstadt, Germany).

**Nuclear extract**

Nuclei were isolated from HeLa cells as previously described (17). Briefly, HeLa cells (2x10⁶) were re-suspended in 4 ml of a 0.25-M sucrose buffer (0.25 mM sucrose, 5.0 mM MgCl₂ and 10 mM Tris, pH 7.4) and, after homogenization at 10 µm clearance using the Isobiotec Cell Homogenizer (Isobiotec Precision Engineering, Heidelberg, Germany), centrifuged for 10 min at 600g. Nuclei were re-suspended in 5 ml of a 2.2-M sucrose buffer (2.2 M sucrose, 5.0 mM MgCl₂ and 10 mM Tris, pH 7.4) and centrifuged for 30 min at 50,000g. Nuclei were then washed in the 0.25-M sucrose buffer and incubated for 1 hr on ice in the same buffer.
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containing 1% (v/v) Triton X-100 (4 ml). A cocktail of protease inhibitors was included in these steps (Sigma-Aldrich). The lipid-depleted nuclei, named TxN, were washed and re-suspended in the 0.25-M sucrose buffer (0.9 ml), before 0.1 ml of 5 M NaCl was added and vigorously mixed by pipetting. The nuclei swelled into a single colloidal gel to which 0.5 ml of the 0.25-M sucrose buffer containing 500 mM NaCl was added. After centrifugation for 10 min at 2,000g, the supernatant was collected. To the pellet, 0.5 ml of the 0.25-M sucrose buffer was added and vigorously mixed by pipetting. After centrifugation, supernatant was again collected. This step was repeated once and all three supernatants were combined as a lipid/chromatin-free nuclear extract, known as TxNE. Its A280 reading is normally 1.0-2.0.

TxN were also adhered to coverslips by incubation for 5 min on ice and then incubated for 10 min on ice with the 0.25-M sucrose buffer containing 100-500 mM NaCl. After fixation, these were immuno-stained for NPM1 and LB1 and analyzed by confocal microscopy.

**Generation of affinity resins**

cDNA for human UBF and histone H1.2 were PCR-amplified from HeLa cell RNA using the following primers: UBF (forward primer, 5' CCGCTAGCATGAAAGAAGAGCCTA'3'; reverse primer, 5'GCAAGCTTTCAGTTGGAGTCAGAGTCTGA3'); and histone H1.2 (forward primer, 5' ATGGCTAGCATGTCGAGACTGCTCCTGC 3'; reverse primer, 5' TTCCGATCCGGTTTATAGGATCCGTT CGC3'). The cDNA fragments were cloned into the pET28 vector between NheI/HindIII (UBF) or NheI/BamHI (H1.2) and the vectors were expressed in E. coli BL-21. The pET28 expression vectors for three H1.2 mutants were synthesized by Genescript (Piscataway, NJ) with flanking 5' NdeI and 3'BamHI restriction sites:

- H1.2
  - NTD (5' GCTAGCTCTCTCCCGGTTCAGA… CGGCGCCAAGAAGAATAGGGATCC 3', amino acid 36-213), H1.2NTD
  - CTD (5' GCTAGCATGTCGAGACTGCTCCTGCG… CCTTTAATCAAGAAGATAGGGATCC 3', amino acid 1-109), and H1.2CTD
  - GD (5' GCTAGCTCTGTCGCCCGGTTCAGA… CTTTTAATCAAGAAGATAGGGATCC 3', amino acid 36-109). Restriction sites are in italic letters and stop codons are underlined.

To purify these recombinant proteins, overnight bacteria cultures (5 ml) were diluted in 50 ml of L-broth containing kanamycin (30 µg/ml) and, when A600 reading reached 0.6-0.8, IPTG was added to 1 mM and further cultured for 3 hr. Bacteria were harvested and sonicated in the binding buffer (50 mM sodium phosphate, 350 mM NaCl, and 10 mM imidazole, pH 7.4) and the supernatant was incubated overnight, in a Poly-prep column (Bio-Rad), with 0.5 ml of Ni-NTA-agarose (Thermo Fisher Scientific). The columns were washed with 30 ml of binding buffer containing 20 mM imidazole and eluted at 150 mM imidazole.

**Affinity chromatography**

Purified UBF and H1.2 were coupled to CNBr-activated Sepharose 4B (GE Healthcare) to generate UBF- and H1.2-Sepharose. The resins were also derived with 1M Tris (pH 8.0) (Tris-Sepharose) to use as a control. TxNE, which contained approx. 300 mM NaCl, was diluted 2 folds in the 0.25-M sucrose buffer (2 ml) and, in a Poly-prep column, incubated with 0.5 ml of UBF-, H1.2-, or Tris-Sepharose for 2 hr at 4°C. The flow through fractions were collected and the columns were each washed with 20 ml of a washing buffer (50 mM Tris, pH 7.4 and 150 mM NaCl). Columns were eluted at 500 mM NaCl (50 mM Tris, pH 7.4 and 500 mM NaCl) in 150-µl fractions. Similarly, purified H1.2 was applied to UBF-Sepharose and purified UBF was applied to H1.2-Sepharose.

**Affinity pulldown of H1 variants and H1.2 mutant proteins**

H1 variants were generated as detailed in Supplemental Figure S3. Three H1.2 mutants were generated by deleting its NTD (H1.2NTD), CTD (H1.2CTD) or both NTD and CTD (H1.2GD). Two hundred microliter of each purified H1 protein or H1.2 mutant (100-200 µg/ml) was incubated with 50 µl of UBF-Sepharose for 2 hr. Supernatants were reserved and the resins were each washed 3 times in the washing buffer. Bound proteins were eluted using 100 µl of 1 M NaCl in the washing buffer. The input, supernatant and elution from each experiment were analyzed by SDS-PAGE and Coomassie blue staining.

**SDS-PAGE and Western blotting**
Samples separated on 12.5% or 18% (w/v) SDS-PAGE gels were either stained with Coomassie blue or electro-transferred for Western blotting. The blots were blocked for 1 hr in TBS-T (50 mM Tris, 150 mM NaCl, pH 7.4, and 0.1% (v/v) Tween 20) containing 5% (w/v) non-fat milk and then incubated overnight at 4°C with different primary antibodies (0.5 µg/ml). After washing in TBS-T, the blots were incubated for 2 hr with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG. Signals were visualized using the PIERCE Supersignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific). Some blots were stripped for 30 min at 50ºC in 62.5 mM Tris (pH 6.8) containing 0.1 M 2-mercaptoethanol and 2% (w/v) SDS and then re-probed with a different antibody.

**LC-MS/MS**

Proteins eluted from UBF-Sepharose were heated for 10 min at 100°C in the presence of dithiothreitol (10 mM) without dye and then alkylated for 30 min at room temperature with iodoacetamide (20 mM) in the dark. Samples were separated on 12.5% (w/v) gels and stained with Coomassie blue. Selected bands were excised and, after trypsin digestion, extracted and analyzed by liquid chromatography tandem mass spectrometry (Experimental Therapeutics Centre, Biopolis Shared Facilities, A-Star, Singapore). Data were analyzed and presented using the Scaffold 4.0.5 software (Proteome Software, Inc., Portland, OR, USA).

**Generation of metaphase cells and isolation of chromosomes**

HeLa cells (80% confluency) were treated with Colcemid (0.1 µg/ml) for up to 24 hr. Cells were harvested at 3-hr intervals in SDS-PAGE sample buffer. After 24 hr, cells were also washed to remove Colcemid and further cultured for 6 or 12 hr. By Western blotting, H1.2, UBF, TopoIIα and LB1 were detected in these cell lysates.

To isolate chromosomes, HeLa cells (~1x10^8) were treated for 15 hr with Colcemid and the detached metaphase cells were washed and re-suspended in the 0.25-M sucrose buffer (2 ml). Cells were homogenized at 18-µm clearance and, after centrifugation for 10 min at 600g, the pelleted chromosomes were re-suspended in the 0.25-M sucrose buffer. To break down the chromosomes, metaphase cells were homogenized at 10-µm clearance. The chromosome fragments were adhered on coverslips by incubation for 10 min on ice and were, after fixation, immuno-stained using mouse anti-UBF and rabbit anti-H1.2 antibodies.

**Confocal microscopy**

HeLa cells cultured on coverslips were fixed for 30 min in 1% (w/v) paraformaldehyde (PFA) and then permeabilized for 1 hr in PBS containing 1% (v/v) Triton X-100. Cells were incubated for 1 hr with mouse anti-UBF and a rabbit antibody for histone H1.2, nucleolin or fibrillarin. Cells were also stained with antibodies for H1.0, H1.1, H1.3, H1.4, H1.5 and H1.x (Supplemental Figure S2). After washing, cells were incubated with goat anti-mouse (Cy3) and anti-rabbit (Alexa Fluor 488 or AF488) IgG. The permeabilized cells were also incubated for 30 min at room temperature with either DNase from the NucleoSpin RNA isolation kit (Machery-Nagel, Duren, Germany) or RNase A at 1 mg/ml in the 0.25-mM sucrose buffer (Qiagen GmbH, Hilden, Germany) before immuno-staining. HeLa cells were also treated for 2 hr with ActD (40 ng/ml) to block Pol I transcription (40,41). These cells were similarly fixed, permeabilized and immuno-stained with a mouse anti-UBF antibody and a rabbit antibody for H1.2 or nucleolin. Cells were washed and mounted using the VectorShield medium containing DAPI and analyzed using a Fluoview FV1000 confocal microscope equipped with a 100x oil objective (aperture 1.45) and Cool/SNAP HQ2 image acquisition camera (Olympus). Images were acquired with the FV-ASW 1.6b software and analyzed using the Imaris software (Bitplane AG).

With TxN, isolated chromosomes, and chromosome fragments, these were first adhered to coverslips by incubation for 10 min on ice and, after fixation, similarly immuno-stained and analyzed by confocal microscopy.

**Scanning electron microscopy**

TxN were adhered to coverslips by incubation for 10 min on ice. After fixing for 2 hr in 2.5% (w/v) glutaraldehyde, the nuclei were washed in PBS and oxidized for 30 min in 1% (w/v) OsO4 (pH 7.4). After washing, the coverslips were dehydrated using increasing concentrations of ethanol (i.e. 50%, 75% and 95% and 100%). The coverslips were then equilibrated and dried in liquid CO2 using the EM CPD 030 Critical Point Dryer (Leica).
Camera AG, Wetzlar, Germany) and gold-coated for 100 sec at 30 mA using a BAL-TEC SCD 005 Sputter Coater (Leica). Samples were analyzed at 10 KV and 20 µA emission current, using the JSM-6701F Field Emission Scanning Microscope (JOEL Ltd, Tokyo, Japan).

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Author contributions
J.C. and B.H.D.T. performed the biochemical experiments. B.H.D.T., Y.C. and J.C. performed most of the fluorescence microscopy experiments. J.C. and J.L. mainly contributed to the ideas and wrote most parts of the manuscript. S.Y.K.W. helped in the transfection experiments. J.L. performed the SEM experiments and finalized the manuscript.

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**Figure legends**

**Figure 1. Extraction of nuclear proteins from the chromatin.** *A*, TxN in the 0.25-M sucrose buffer were made 100-500 mM with NaCl. After vigorously pipetting and centrifugation, supernatants were analyzed by SDS-PAGE Coomassie blue staining. Cytosol, isolated nuclei, and TxN were also included. *B-K*, Cytosol, nuclei, TxN and the nuclear extracts were probed by Western blotting with mouse and rabbit antibodies specific for nucleolar (NPM1, fibrillarin, NCL and UBF), nuclear lamina (lamins A, C and B1), and some chromatin-associated (cenP-A, HP1γ, RNA pol II and TopoIIα) proteins. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG were used as secondary antibodies. *L and M*, TxN on coverslips were exposed to 500 mM NaCl for 15 min, fixed, and stained for lamin B1 (AF488, green), NPM1 (Cy3, red), and chromatin (DAPI, blue). *L*, chromatin. *M*, merged signals. Scale bar, 5 µm. *N and O*, NaCl-extracted TxN on coverslips were processed for SEM analysis. Images were shown at two different magnifications: *N*, x70,000. *O*, x160,000. The square in *N* is equivalent to the image in *O*.

**Figure 2. Histone H1.2 is a novel UBF-binding protein.** *A*, TxNE (1 ml) was 1:1 diluted with 50 mM Tris (pH 7.4) and incubated with UBF-Sepharose (0.5 ml) for 2 hr at 4°C in a column. The column was washed and eluted at 500 mM NaCl. The first 7 fractions, the input TxNE, and the flow through were analyzed by SDS-PAGE and Coomassie blue staining. *B*, diluted TxNE (2 ml) was incubated with 0.5 ml of H1.2-Sepharose and, as a control, Tris-Sepharose. After elution, fractions 4-6 were combined (elution). The input TxNE, the flow through fraction, and the elution were analyzed by Western blotting using anti-UBF and anti-HMGB1 antibodies. *C*, recombinant H1.2 (2 ml) was incubated with UBF-Sepharose and bound proteins were eluted. Input H1.2, the flow through, and eluted fractions were analyzed by SDA-PAGE Coomassie blue staining (upper panel) and Western blotting (lower panel). *D*, recombinant UBF (2 ml) was incubated with H1.2-Sepharose. The input UBF, the flow through, and the eluted fractions were analyzed by SDS-PAGE (upper panel) and Western
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Histone H1.2 recruitment to NORs (lower panel). E, the three purified H1.2 mutants H1.2ΔNTD, H1.2ΔCTD and H1.2GD (200 µl) were each incubated with 50 µl of UBF-Sepharose for 2 hr. Protein input, the absorbed supernatants, and the elution were compared by SDS-PAGE Coomassie blue staining. Samples were examined on 18% (w/v) gels. F, purified H1.1, H1.3, H1.4, H1.5 and H1.x were similarly incubated with UBF-Sepharose or, as a control, Tris-Sepharose (H1.2). The input proteins, absorbed supernatants and elution were compared on 12.5% (w/v) gels.

Figure 3. Cellular distribution of UBF and histone H1.2 in interphase and mitotic cells. HeLa cells were fixed, permeabilized and incubated first with mouse anti-UBF and rabbit anti-H1.2 antibodies and then with secondary goat anti-mouse (Cy3, red) and anti-rabbit (Alexa Fluor 488 or AF488, green) IgG. Cells were mounted with a DAPI-containing medium and analysed by confocal microscopy. Images were captured as 0.36-µm serial sections. Using the Imaris software, 3D images were re-constructed for the mitotic images. Scale bars, 5 µm.

Figure 4. Dispersion of nucleolin and fibrillarin from mitotic NORs. Cells on coverslips were stained with mouse anti-UBF and rabbit anti-nucleolin or anti-fibrillarin antibodies, followed by secondary goat anti-mouse (Cy3, red) and anti-rabbit IgG (AF488, green). After mounting using a DAPI-containing medium, interphase and prophase cell images were captured by confocal microscopy in 0.36-µm serial sections. 3D images were re-constructed using the Imaris software. Single and merged images are presented. Scale bars, 5 µm.

Figure 5. Inhibition of rRNA transcription in interphase nuclei causes H1.2 condensation into NOR-like structures. Cells cultured on coverslips were treated with ActD (40 ng/ml) for 2 hr and then fixed, permeabilized and stained using mouse anti-UBF and rabbit anti-H1.2 or anti-nucleolin (NCL) antibodies. Cells were then stained with secondary goat anti-mouse (Cy3, red) and anti-rabbit IgG (AF488, green). After mounting with a DAPI-containing medium, cells were analysed by confocal microscopy in 0.36-µm serial sections. 3D images were re-constructed using the Imaris software. Single and merged signals. Scale bars, 5 µm.

Figure 6. Sustained H1.2 expression during interphase and mitosis and the structural independence of NORs. A, HeLa cells were treated for up to 24 hr with Colcemid (0.1 µg/ml) and harvested at 3-hr intervals to generate cell lysates. After 24 hr, Colcemid was removed. Cells were further cultured for 6 and 12 hr. Sample loading was normalized based on total cellular proteins (top panel). UBF, H1.2, lamin B1 and TopoIIα levels were determined by Western blotting. Arrow head, possible UBF fragment. B-G, HeLa cells treated with Colcemid (0.1 µg/ml, 15 hr) were homogenized. After centrifugation, chromosomes were harvested in the pellets. On coverslips, these chromosomes were fixed and stained with mouse anti-UBF (Cy3, red), rabbit anti-H1.2 (AF488, green), and DAPI (blue) and analysed by confocal microscopy. 3D images were re-constructed from the 0.36-µm serial section images collected. B-D, single signals. E-G, merged signals. Two prominent NORs are highlighted by squares and presented at higher magnifications. Scale bar, 5 µm.

Figure 7. Revelation of chromosome-associated H1.2 after DNase digestion. HeLa cells on glass coverslips were fixed and permeabilized. Upper panels, cells were incubated with the DNase buffer for 30 min before staining with rabbit anti-H1.2 and mouse anti-UBF antibodies. Cells were then stained using secondary goat anti-rabbit (AF488) and anti-mouse (Cy3) IgG. Middle panels, permeabilized cells were incubated with DNase for 30 min before immuno-staining. Lower panels, permeabilized cells were treated with RNase A for 30 min before immuno-staining. Coverslips were mounted with a DAPI-containing medium and serial 0.36-µm section images were captured by confocal microscopy. 3D images were re-constructed using the Imaris software. Scale bars, 5 µm.
Figure 8. NORs are separable from chromosomes. A-F, HeLa cells were treated with Colcemid for 15 hr and homogenized at 10-µm clearance. After centrifugation, pelleted materials were adhered to coverslips and fixed. These were stained first with mouse anti-UBF and rabbit anti-H1.2 antibodies and then stained with secondary goat anti-mouse (Cy3, red) and anti-rabbit (AF488, green) IgG. After mounting with DAPI-counting medium, serial 0.36-µm section images were captured by confocal microscopy. 3D images were reconstructed using the Imaris software. A-C, single signals. D-F, merged signals. G-I, HeLa cells were treated for 15 hr with Colcemid on coverslips and then fixed and stained for UBF, H1.2 and the chromatins. Merged 3D images are shown: G, H1.2 and chromatins, H, UBF and chromatins, I, H1.2 and UBF. Scale bars, 5 µm.

Figures

Figure 1
Figure 2
Figure 3

| Interphase | Prophase | Promataphase | Metaphase | Anaphase | Telophase |
|------------|----------|--------------|-----------|----------|-----------|
| Chromatin  |          |              |           |          |           |
| UBF        |          |              |           |          |           |
| H1.2       |          |              |           |          |           |
| UBF/H1.2   |          |              |           |          |           |

DAP (blue)  
Cy3 (red)   
AF488 (green)  
Cy5/AF488  

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Figure 4

![Images showing DAPI, UBF, NCL, and Merge](image1)

Figure 5

![Images showing DAPI, Histone H1.2, UBF, and Histone H1.2/UBF](image2)
Figure 6

Figure 7
Figure 8
The linker histone H1.2 is a novel component of the nucleolar organizer regions
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