Vertebrate nucleoplasmin and NASP: egg histone storage proteins with multiple chaperone activities

Ron M. Finn,*[,1] Katherine Ellard,*[,1] José M. Eirín-López,[†] and Juan Ausió*[,2]

*Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, Canada; and [†]Chromatin Structure and Evolution (CHROMEVOL) Group, Departamento de Biología Celular y Molecular, Universidade da Coruña, A Coruña, Spain

ABSTRACT **Recent reviews have focused on the structure and function of histone chaperones involved in different aspects of somatic cell chromatin metabolism. One of the most dramatic chromatin remodeling processes takes place immediately after fertilization and is mediated by egg histone storage chaperones. These include members of the nucleoplasmin (NPM2/NPM3), which are preferentially associated with histones H2A-H2B in the egg and the nuclear autoantigenic sperm protein (NASP) families. Interestingly, in addition to binding and providing storage to H3/H4 in the egg and in somatic cells, NASP has been shown to be a unique genuine chaperone for histone H1. This review revolves around the structural and functional roles of these two families of chaperones whose activity is modulated by their own post-translational modifications (PTMs), particularly phosphorylation. Beyond their important role in the remodeling of paternal chromatin in the early stages of embryogenesis, NPM and NASP members can interact with a plethora of proteins in addition to histones in somatic cells and play a critical role in processes of functional cell alteration, such as in cancer. Despite their common presence in the egg, these two histone chaperones appear to be evolutionarily unrelated. In contrast to members of the NPM family, which share a common monophyletic evolutionary origin, the different types of NASP appear to have evolved recurrently within different taxa.—Finn, R. M., Ellard, K., Eirín-López, J. M., Ausió, J. Vertebrate nucleoplasmin and NASP: egg histone storage proteins with multiple chaperone activities. *FASEB J.* 26, 4788–4804 (2012). www.fasebj.org**

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In 1978, Ron Laskey coined the term "molecular chaperone" to describe the ability of nucleoplasmin to assist in the formation of nucleosomes by preventing the aggregation of core histones with DNA (1). A decade later, R. J. Ellis expanded on the "molecular chaperone" concept to include proteins that assist in the post-translational assembly of other protein complexes (2). Before chaperone functions were understood, it was believed that proteins folded and arranged themselves into larger macromolecular complexes in a spontaneous, independent way, requiring little additional assistance (3). However, over the past 4 decades, the roles of protein chaperones have been clarified, and they are now known to play an integral part in the formation of manifold cellular complexes. The term "chaperone" is now used to describe an immense and varied compendium of proteins in both eukaryotes and prokaryotes that assist in the post-translational processing of proteins to ensure proper folding and protein complex formation. Nowhere is this more apparent in the context of a cell, than in the extensive and diverse range of histone chaperones involved in the rapid assembly and disassembly of chromatin.

Chromatin is a vast, highly dynamic and yet remarkably organized assemblage of DNA and proteins found in all eukaryotic cells. It allows for the compaction of a large quantity of DNA into the small nucleus of a cell, where it organizes and protects DNA through the formation of higher-order chromatin structures. The rearrangement of these higher-order chromatin structures allows for rapid structural transitions in order to

Abbreviations: AML, acute myeloid leukemia; ASF1, anti-silencing function 1; CAF-1, chromatin silencing factor 1; CCD, coiled-coiled domain; CENP-A, centromere protein A; DAXX, death-associated protein 6; DEK, *Drosophila* Eph kinase; DSB, double-strand break; HAT, histone acetyltransferase; Hif1, Hat1p interacting factor-1; HIRA, histone cell cycle regulation defective homolog A; Hsp90, heat-shock protein 90 kDa; K_d, dissociation constant; NAP1, nucleosome assembly protein 1; NASP, nuclear autoantigenic sperm protein; NES, nuclear export signal; NLB, nucleolus-like body; NLP, nucleoplasmin-like protein; NLS, nuclear localization signal; NPM, nucleoplasmin; NPM1, nucleophosmin; NPM2, nucleoplasmin; NPM3, nucleophosmin/nucleoplasmin 3; PTM, post-translational modification; RD, replication-dependent; RI, replication-independent; SHNi-TPR, Sim3-Hif1-NASP interrupted TPR; Sim3, silencing in the middle of the centromere protein 3; sNASP, somatic NASP; SPR, surface plasmon resonance; tNASP, testes-specific NASP; TPR, tetratricopeptide repeats

[1] These authors contributed equally to this work.

[2] Correspondence: Department of Biochemistry and Microbiology, University of Victoria, Petch building, 258a, Victoria, BC, Canada V8W 3P6. E-mail: jausio@uvic.ca

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adjust to a variety of genomic processes, such as DNA replication, transcription, recombination, and repair.

The principal repeating unit of chromatin is the nucleosome, composed of a histone octamer containing an H3/H4 tetramer and two H2A/H2B dimers, around which 147 bp of DNA are wrapped in ~1.67 left-handed superhelical turns (4). Nucleosomes form "beads on a string" arrays composed of nucleosome core particles connected by varying lengths of linker DNA onto which linker histones (histone H1 and H5 family) and other nonhistone chromatin-binding proteins are associated to construct higher-order chromatin structures (5–7). With the exception of particular regions of the genome undergoing specific genomic processes, such as actively transcribing genes or during DNA repair, for example, all DNA in a cell is assembled into nucleosomes or similar structures.

The assembly and disassembly of nucleosomes must occur in a stepwise fashion, as new histones are deposited onto newly synthesized DNA or as old histones are replaced by new histones and/or histone variants, during the rest of the cell cycle. All of this is facilitated through histone transport, deposition, eviction, and storage (8, 9). Such essential processes are often induced and mediated by a number of ATP-dependent chromatin remodeling complexes, nonhistone chromatin-associated proteins, post-translational modification enzymes, and histone chaperones (10). The latter are often found interacting with the nucleosomes during key cellular processes (11), and the loss of their chaperone function can result in DNA damage, genomic instability, cell cycle arrest, or cell death (12–15).

To further underscore the importance of histone chaperones in the rapid restructuring of nucleosomes, one has to consider that this assembly and disassembly activity has to be accomplished accurately and in a timely fashion in the context of intricate nucleosome structures. These structures are highly stable complexes composed of >120 direct protein-DNA interactions and several hundred water- and ion-mediated interactions (4, 16). In addition, it should be noted that chromatin structure varies across the genome, depending on numerous factors, such as whether a gene is being actively transcribed, or whether the region of chromatin is within an exon, intron, or another element, such as a promoter region for a gene and nucleosomal organization for that particular region. The location on the chromosome is also important, as chromatin near or on the centromere or telomere of a chromosome is substantially different from that on other regions of the chromosome. Structural differences are even observed across different cell types, as the composition of chromatin from germ cells differs significantly from that of somatic, transformed, and embryonic stem cells.

In this review, we focus our attention on two vertebrate histone chaperones [nucleoplasmin (NPM) and nuclear autoantigenic sperm protein (NASP)] that are massively present in the egg and are thus potentially involved in chromatin remodeling during early stages of embryogenesis. In addition, they participate in many other aspects of nuclear metabolism. In particular, this is the first comprehensive review on NASP, a member of the silencing in the middle of the centromere protein 3 (Sim3)–Hat1p-interacting factor-1 (Hif1)–NASP-interrupted tetratricopeptide repeats (TPR) family (SHNi-TPR family; also known as the N1/N2 family; ref. 17). Interestingly, in contrast to most of the histone chaperones characterized to date, NASP exhibits a unique ability to bind to linker histones of the H1 family in addition to histones H3 and H4.

HISTONE CHAPERONES

In somatic cells, extensive networks of histone chaperones work synergistically to deposit core and linker histones onto DNA, which results in the formation of nucleosomes and higher-order chromatin structures (18–23). Histone chaperone research has primarily focused on the characterization of processes involved in the stepwise addition of core histones during nucleosome formation in somatic cells, while less is known about the chaperones involved in chromatin remodeling processes that take place following egg fertilization. In the eggs of vertebrate organisms, the chaperone responsible for the storage of H3/H4 and possibly H1 is NASP (SHNi-TPR family) and for H2A/H2B is NPM family. This review will focus on these particular groups of histone chaperones, with particular emphasis on *Homo sapiens* nucleophosmin (NPM1), nucleoplasmin (NPM or NPM2), and nucleophosmin/nucleoplasmin 3 (NPM3) and on the two primary *Homo sapiens* isoforms of NASP, testes-specific NASP (tNASP) and somatic NASP (sNASP).

Before newly synthesized histones can be incorporated into chromatin, they must first be produced in the cytoplasm and protected from protein degradation and nonspecific interactions. In addition, appropriate histone levels must be strictly regulated on a translational and post-translational level to prevent genomic instability or abnormal chromatin structures (24). The highly positive nature of histones can result in free histones binding nonspecifically to DNA, RNA, and negatively charged proteins if they are present in excess and has the potential to cause cellular cytotoxicity due to genome instability, DNA damage, aberrant protein-protein interactions, and delays in cell cycle progression, cell senescence, or cell death (12–15). To prevent these aberrant associations, chaperone proteins bind to histones as they are produced, abrogating any undesirable interactions and poising them for transfer to other chaperones in replication-dependent (RD) and replication-independent (RI) pathways, as required (1, 18, 25–27). A great deal remains to be determined about the processes involved after the synthesis of histones, their transport into the nucleus, and their subsequent association with the chaperones that directly interact with chromatin silencing factor 1 (CAF-1) complex (RD pathway) and histone cell cycle regulation defective

homologue A (HIRA) complex or death-associated protein 6 (DAXX)/ *Drosophila* Eph kinase (DEK) complexes (RI pathway). Much of what is known about the steps involving H3/H4 incorporation into the nucleosome has served as a model for how histone chaperone networks work (18–23, 28). Focus has been on the events that follow the binding of H3.1/H4 or H3.3/H4 to antisilencing function 1 (Asf1; refs. 28, 29). Asf1 controls the replication fork advancement and interacts with old histones evicted from nucleosomes and newly synthesized histones that are in transit on their way to be incorporated into newly formed nucleosomes (30). Asf1 subsequently transfers replication-dependent H3/H4 dimers to the CAF-1 complex, which participates in the assembly of nucleosomes by incorporating H3/H4 tetramers onto DNA in a process that is coupled to DNA synthesis (28–30). Asf1 also participates in the assembly of nucleosomes in a replication-independent manner by the handing off of replication-independent variant H3.3/H4 dimers to the HIRA complex or the newly characterized DAXX and DEK complexes (18). H2A/H2B dimers are either directly or indirectly incorporated onto the H3/H4 tetramer by facilitates chromatin transcription (FACT; ref. 31) and nucleosome assembly protein 1 (NAP1; ref. 32) to form the nucleosomes. While the steps following Asf1, Nap1, and, to some extent, FACT's role in nucleosome assembly have been the primary focus of research, it has come to light that the NPM and NASP family of chaperones play important roles in the preceding steps, leading from histone synthesis and storage to the formation of nucleosomes. This finding includes the possibility that under the right cellular context or cell types, the NPM and NASP family of chaperones can substitute for the aforementioned chaperones and directly or indirectly assemble or disassemble nucleosomes not only *in vitro* (33–34) but also *in vivo*.

NPM FAMILY OF HISTONE CHAPERONES

The founding member of the NPM family, nucleoplasmin, was initially labeled as the archetypal molecular chaperone. This family of histone chaperone proteins can be categorized into 3 main groups: NPM1, NPM2, and NPM3, that are expressed throughout eukaryotes, sharing similarities both in structure and amino acid sequence (**Fig. 1**). Members of this family have been shown to play a role in a large variety of cellular processes (**Fig. 2**) and are essential for both chromatin remodeling following fertilization *in vivo* (35, 36) and nucleosome assembly/disassembly in germinal and somatic cells (37).

The most extensively studied member of this family, NPM1 (also known as B23, numatrin, and NO38), is a well-characterized protein ubiquitously expressed throughout different types of cells. The abundance of research focusing around NPM1 is largely due to the fact that it commonly undergoes genetic alterations in acute myeloid leukemia. A nucleolar phos-

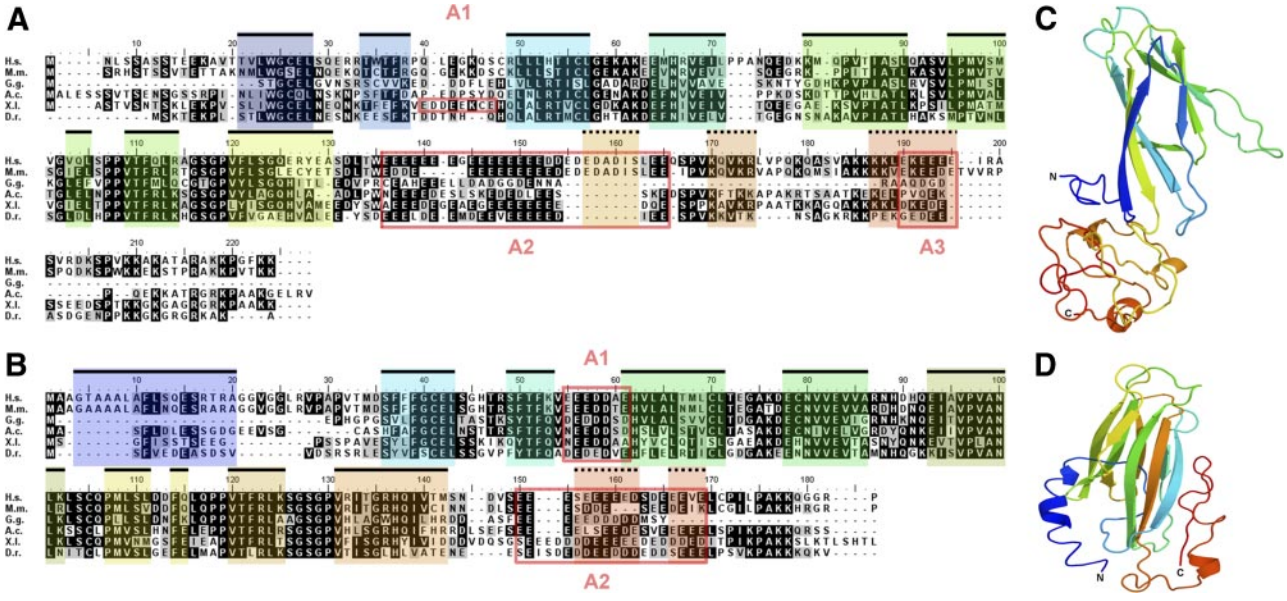

Figure 1. *A*, *B*) Amino acid sequence alignment of NPM2 (*A*) and of NPM3 (*B*) proteins in different vertebrate species. Conserved residues are white over a black background, and similar residues are white over a gray background. Horizontal bars over amino acids indicate regions of β-strand structure, and dotted lines over amino acids indicate region of α-helical structure. H.s., *Homo sapiens* (NPM2: NP_877724.1, NPM3: NP_008924.1); M.m., *Mus musculus* (NP_851990.2, NPM3: NP_032749.1); G.g., *Gallus gallus* (XP_001233986.1, NPM3: XP_001233764.2); A.c., *Anolis carolinesis* (XP_003225756.1, NPM3: XP_003224272.1); X.l., *Xenopus laevis* (NP_001081027.1, NPM3: NP_001087275.1); D.r., *Danio rerio* (NP_001116479.2, NPM3: NP_001013502.1). Red boxes indicate acidic tracts A1, A2, and A3. *C*, *D*) Predicted structure of *H. sapiens* NPM2 (*C*) and of NPM3 (*D*) determined by Phyre 2 server (http://www.sbg.bio.ic.ac.uk/~phyre/), displaying 50% of amino acid sequence with 90% confidence. The NPM2 prediction was based on the recently determined partial crystallographic structure (N-terminal domain) of human NPM2 (90). Colors in the 3D structures correspond to those highlighted in the sequences shown in *A*, *B*.

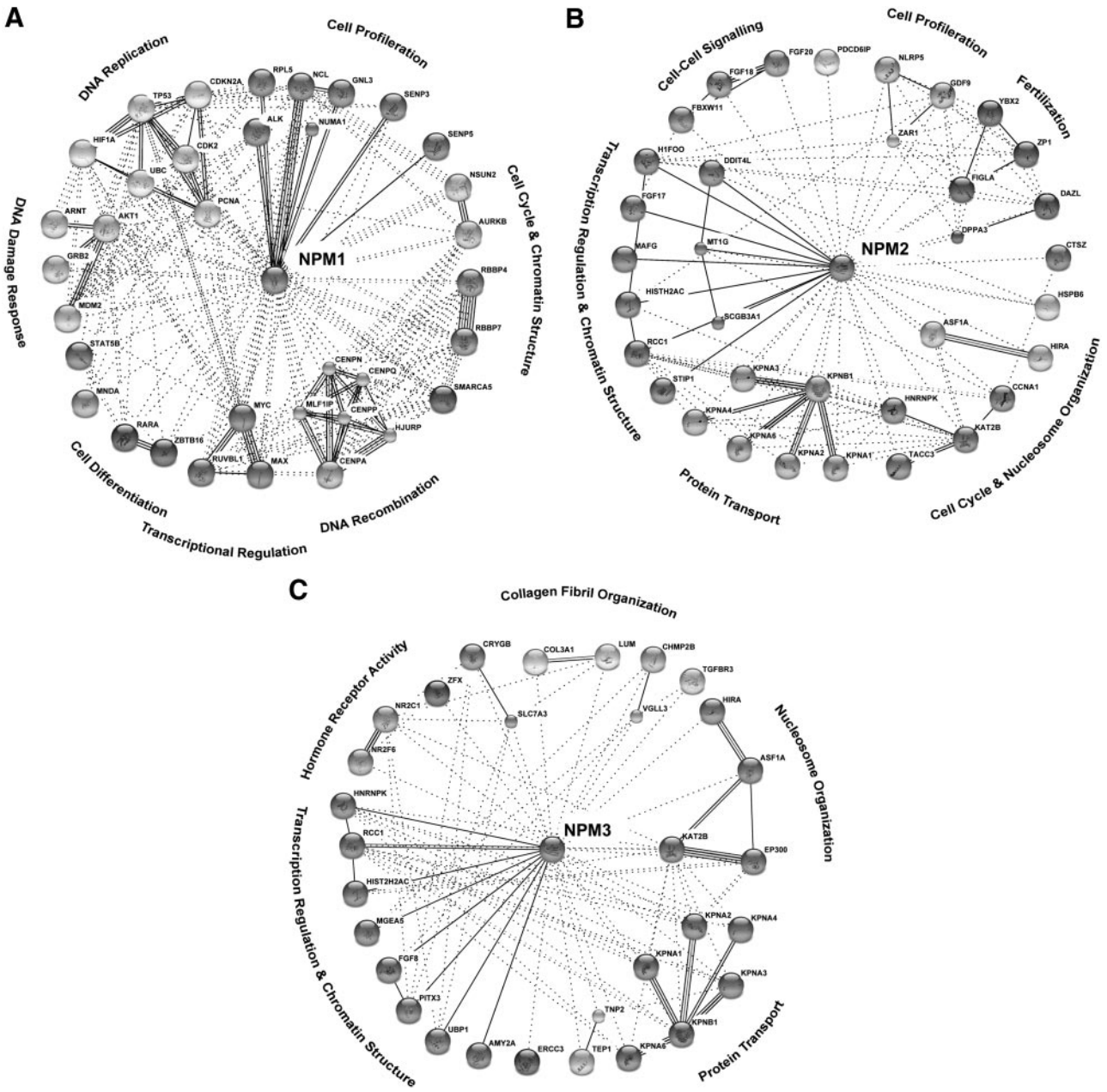

Figure 2. Protein interactome of *H. sapiens* NPM1 (*A*), NPM2 (*B*), and NPM3 (*C*) generated using STRING 9.0 (ref. 124; http://string-db.org) displayed in evidence view with a custom-required confidence score of 0.5 and a limitation of 50 interactions. Solid lines represent direct evidence for the association: proteins that are associated by experimental, fusion, membership in a complex, cooccurrence, or coexpression evidence; dotted lines connect proteins that are associated by text mining or database evidence (124). To allow for functional associations between groups of proteins to be visualized, protein nodes were clustered using k-means algorithm (with an input parameter of 10) into different node clusters according to their distance matrix values from their String 9.0 global scores. To provide clarity, proteins may be categorized under a particular biological function heading; however, they may be involved in several other biological processes listed and in other processes not listed in the figure.

phoprotein, NPM1 has been linked to having many diverse roles within the cell, including ribosomal biogenesis (38), partly based on its localization with preribosomal ribonucleoprotein particles and its ability to facilitate ribosome assembly (39); genomic stability, as inactivation of the NPM1 gene in mice led to noticeable defects in centrosome duplication (40); DNA replication (41), transcriptional regulation (42), histone chaperone activity (43), and nucleic acid binding (44) (Fig. 2*A*). The human *NPM1* gene is a 23.8-kb gene comprising 10 exons; when spliced, at least 19 variants result, with 13 of these having the potential to encode proteins (45).

The second member of this chaperone family, NPM2, was found to be the most abundant protein in the egg of *Xenopus laevis*, where it serves as a storage protein for H2A/H2B that will be deposited onto sperm chromatin following fertilization (1, 26, 27,

46–48). NPM2 in *X. laevis* assists both in the removal of protamines (49) and the subsequent deposition of H2A/H2B dimers, highlighting its important role in chromatin decondensation and proper nucleosome formation (refs. 1, 50 and Fig. 2*B*). In addition, NPM2 displays an ability to decondense chromatin of introduced somatic cell nuclei (51), but it is explicitly expressed in mature oocytes and eggs (52), contrary to other NPM family members. Acting as a reservoir for H2A/H2B dimers within oocytes, NPM2 binds to 5 H2A-H2B dimers in its oligomer form (53, 54). However, the exact binding sites of protamines and histones with NPM2 is still controversial, as they have been suggested to bind to both the distal (53) and lateral (54–56) face of the pentameric protein. The human *NPM2* gene is a 1.1-kb gene comprising 10 exons (**Fig. 3*A***) that is alternatively spliced and predicted to have at least 11 splice variants, 9 of these variants potentially encoding proteins (45), with the predominant transcript producing a 214-aa protein (Fig. 3*B*).

Recently, it was demonstrated that NPM3, the least fully characterized member of the NPM family, interacts with NPM1 and subsequently alters pre-rRNA synthesis and processing (57), supporting earlier findings that NPM1 actively participates in ribosomal biogenesis (58). Through a diverse range of biological functions, the NPM chaperone family possesses a critical role in DNA replication, histone storage, and histone chaperone activity (Fig. 2). The human *NPM3* gene is a 2.1-kb gene comprising 10 exons that is alternatively spliced and predicted to have at least 5 splice variants, with 3 of these variants potentially encoding proteins (45).

NASP AND THE SHNi-TPR FAMILY OF HISTONE CHAPERONES

First characterized in rabbit testes as a sperm/testes-specific protein that was highly autoantigenic, the mammalian NASP was found to have a high degree of sequence similarity to a NASP homologue in *X. laevis*, N1/N2 (59, 60), present in most vertebrates as a somatic and a testes-specific isoform (**Figs. 4** and **5**, respectively). N1/N2 interacts specifically with H3/H4 (46–48, 61), providing a novel mechanism of storage for these histones (46, 62, 63). NASP is widely distributed throughout eukaryotes in a wide range of tissue types, with multiple isoforms present in the same species, as supported by biochemical and phylogenetic analysis (33, 64). NASP is either directly or indirectly essential for eukaryotic DNA replication (14, 65–72), cell proliferation (70, 73), normal cell cycle progression (14, 65–74), blastocyst development (14, 65–72), cellular growth (70, 73), histone storage (1, 18, 25–27), histone transport (33, 34, 65, 74, 75), stem cell proliferation (76), neural stem cell differentiation (77), and the pluripotency of human embryonic stem cells (hESCs; ref. 76) (**Fig. 6**). In mammals, there are commonly two predominant isoforms, one significantly smaller (Fig. 4) than the other (Fig. 5), the result of alternative splicing of the coding region of the gene. The human *NASP* is a 35.33-kb gene comprising 15 exons (**Fig. 7*A***) that is alternatively spliced and predicted to have at least 26 splice variants, with 20 of those variants potentially encoding proteins (45), although only two of those variants have been characterized in *in vivo* and *in vitro* studies, thus far. The two predominant human mRNA transcripts differ in their 5′-untranslated

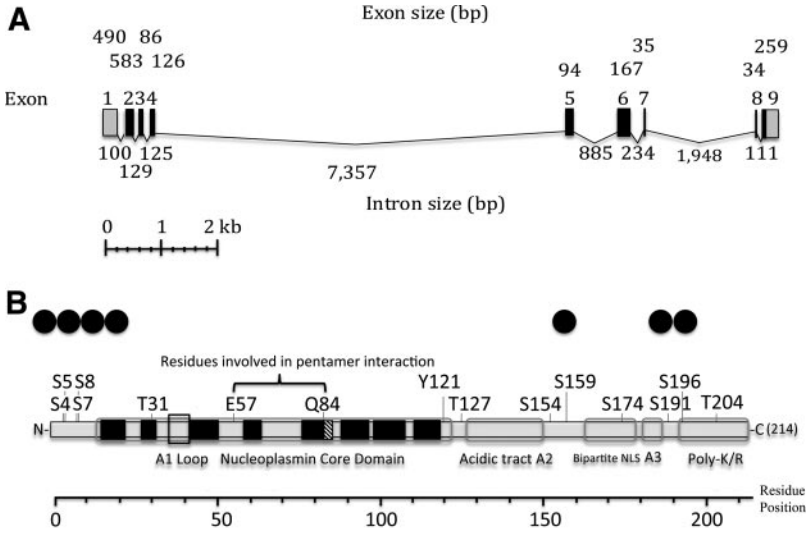

Figure 3. *A*) Schematic representation of the exon and intron organization of the *H. sapiens* NPM2 gene (NCBI GeneID: 10361). Solid black boxes represent exons coding for translated regions; shaded boxes represent exons coding for untranslated regions. Solid black lines indicate introns. Size of exon in base pairs is indicated above each exon and below each intron. *B*) Schematic diagram of *H. sapiens* NPM2 (UniProtKB: Q86SE8) displaying the predicted secondary structure, selection of functional motifs and residues, and phosphorylation sites. Solid black boxes represent secondary β-strand structure, striped box represents a secondary "turn" structure. Outlined in shaded boxes: nucleoplasmin core domain, conserved in all NPMs; acidic A2 and A3 tracts that contain numerous glutamic and aspartic acid residues; acidic tract A1 present in *X. laevis* NPM2 is absent in *H. sapiens* NPM2. Alternatively, this region is referred to as the A1 loop, according to Platonova *et al.* (89). Also indicated, the bipartite NLS, which targets NPM2 for nuclear import, and the polyarginine and polylysine C-terminal tract. Along the top half of the schematic, the two residues involved in pentamer interaction (89) are indicated, E57 and Q84. Also shown are residues that have been experimentally determined to be phosphorylated or predicted to be phosphorylated by the NetPhos 2.0 server (125); phosphorylated residues are labeled by type of amino acid and residue number: serine (S#), threonine (T#), and tyrosine (Y#). Only those residues scoring with a high confidence score on NetPhos 2.0 (above the threshold value of 0.5), and therefore likely phosphorylation sites, are indicated. Approximate locations of the experimentally determined sites in *X. laevis* NPM2 are indicated by large solid black circles for phosphorylated serine (91).

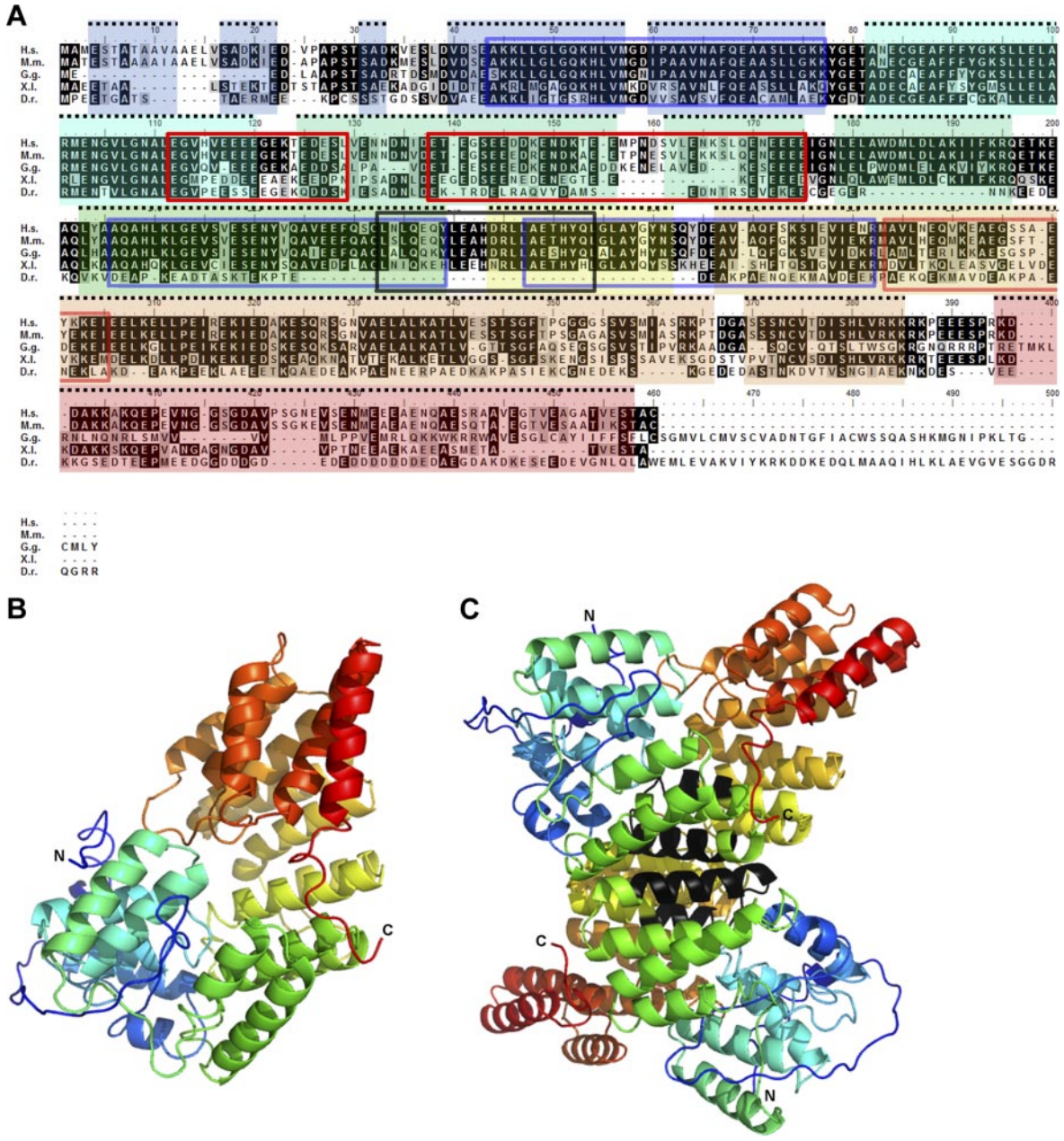

Figure 4. *A*) Amino acid sequence alignment of sNASP proteins in different vertebrate species. For a detailed description, see Fig. 1*A*. H.s., *H. sapiens* (NP_689511.2); M.m., *M. musculus* (NP_001074944.1); G.g., *G. gallus* (CAG30998.1); X.l., *X. laevis* (AAH77440.1); D.r., *D. rerio* (AAH68344.1) The only NASP isoform identified, thus far, for *A. carolinesis* was larger than the sNASP and, therefore, incorporated with the alignments for tNASP isoforms in Fig. 5. Red boxes indicate putative histone-binding motifs, TPRs are indicated by blue boxes, and the putative leucine zipper motif is indicated by a black box. *B*) Predicted structure of *H. sapiens* sNASP, determined by Phyre 2 server (http://www.sbg.bio.ic.ac.uk/~phyre/), displaying 88% of amino acid sequence with 90% confidence. *C*) Illustration of sNASP dimer structure based on predicted structure from *B* and involving the putative leucine zipper motifs indicated in black.

regions, and their alternative splicing results in two deletions in the coding region of NASP, yielding two isoforms, tNASP and sNASP (ref. 74 and Fig. 7*B*). These two NASP isoforms possess histone H3/H4 chaperone activity (18, 25, 34, 74) and have demonstrated histone H1 chaperone activity (33, 65, 74), establishing them as crucial players in the final or last stages of the assembly of chromatin after DNA replication (70). Expression of sNASP mRNA is regulated during the cell cycle, and it parallels the histone mRNA expression (60, 73), as cell cycle progression is delayed when sNASP is overexpressed (68) and delayed along with DNA replication when sNASP is underexpressed (70).

NASP has a strong sequence identity and structural similarity to a family of previously uncharacterized proteins that are now known to function as histone chaperones, the SHNi-TPR superfamily (17). Members of this family have been found in yeast, where their H3/H4 chaperone activity has been demonstrated both *in vivo* and *in vitro*. These NASP ho-

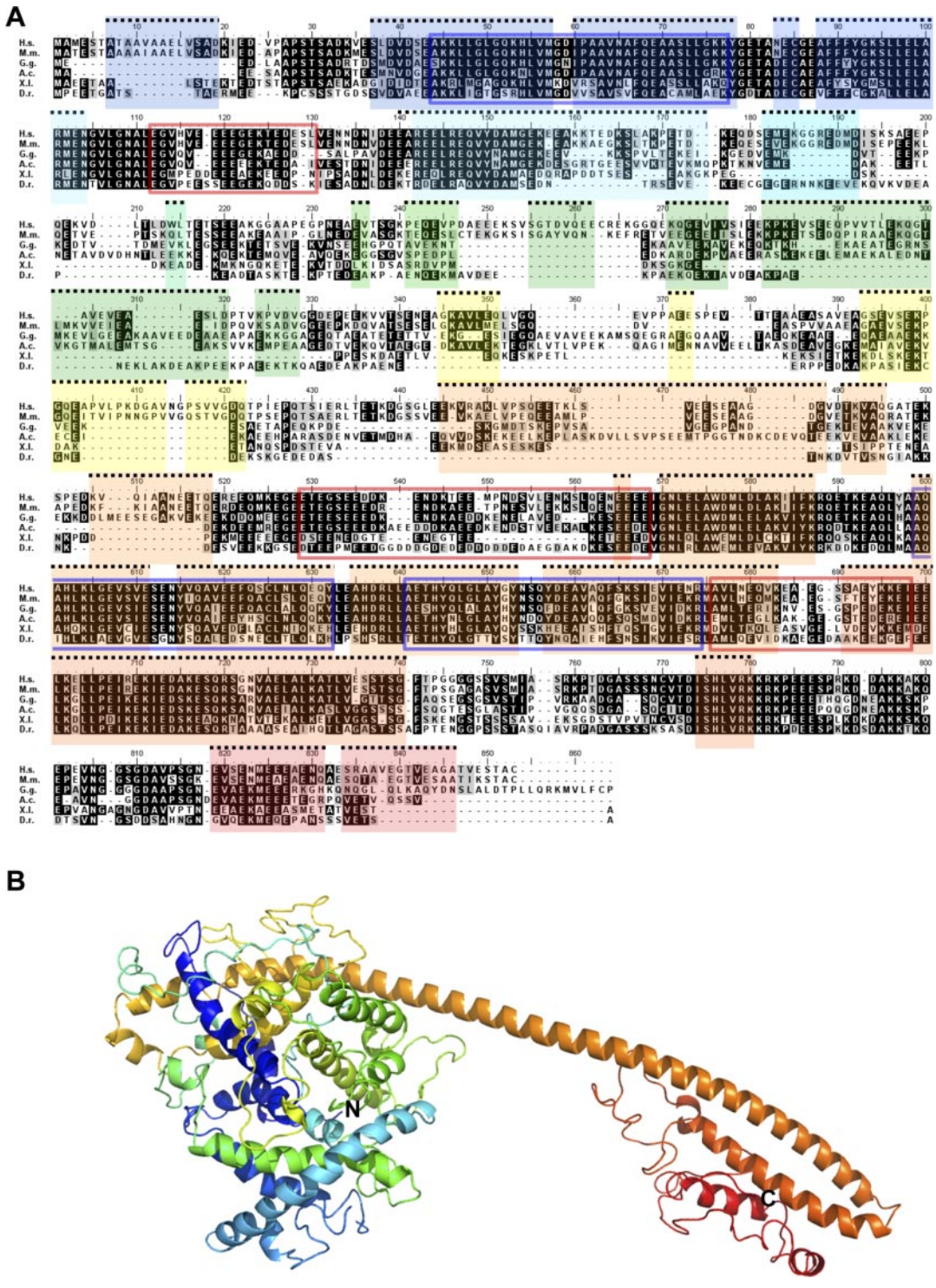

Figure 5. *A*) Amino acid sequence alignment of tNASP proteins in different vertebrate species. H.s., *H. sapiens* (NP_002473.2); M.m., *M. musculus* (NP_058057.3); G.g., *G. gallus* (XP_001235060.2); A.c., *A. carolinesis* (XP_003220306.1); X.l., *X. laevis* (AAI70018.1); D.r., *D. rerio* (NP_956076.1). For a detailed description, see Fig. 1*A*. *B*) Predicted structure of *H. sapiens* sNASP, determined by Phyre 2 server (http://www.sbg.bio.ic.ac.uk/~phyre/), displaying 95% of amino acid sequence with 37% confidence.

molog include a *Schizosaccharomyces pombe* chaperone, Sim3 (17), that is essential for the deposition of centromere protein A (CENP-A) at the centromeres, and yeast without functioning Sim3 have chromosome segregation errors, a role further supported by sNASP's ability to chaperone CENP-A *in vitro* (34). The related *Saccharomyces cerevisiae* chaperone Hif1p is able to bind H3/H4 and facilitate the formation of a nucleosome *in vitro* (61, 74, 75). Hif1p is also essential for H3-specific histone acetyltransferase (HAT) activity during DNA repair in *S. cerevisiae* as a consequence of altering chromatin structure by its

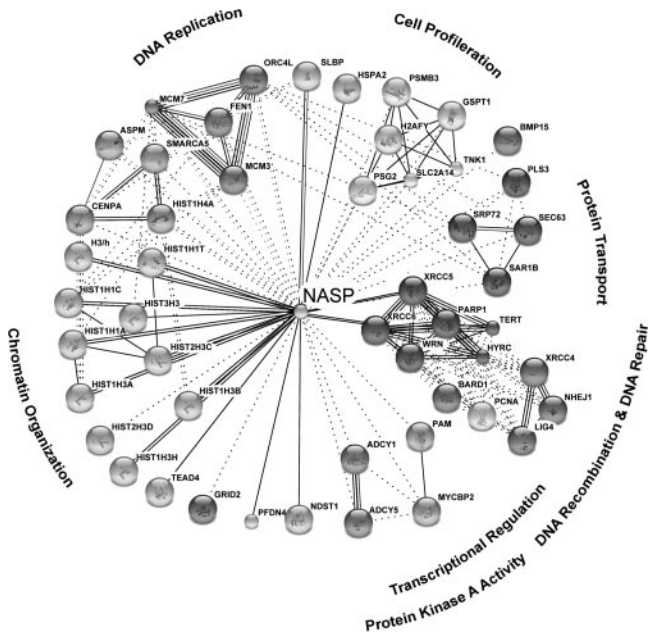

Figure 6. Protein interactome of *H. sapiens* NASP. Node network of predicted associations between proteins based on user-generated input of a protein sequence. Generated using STRING 9.0 (ref. 124; http://string-db.org) displayed in evidence view with a custom-required confidence score of 0.5 and a limitation of 50 interactions. For more details regarding the connecting lines, see the legend to Fig. 2.

H3 chaperone activity and allowing HAT-B complex members, Hat1p-Hat2p, to access their H3 substrate (78). Its ability to alter chromatin also explains deletion of Hif1p, only resulting in a decrease in H3-specific HAT activity (78) and defects in DNA double-stranded breaks (DSBs) and telomere silencing (61, 79). Both sNASP and Hif1p were able to functionally substitute for each other in *in vitro* nucleosome assembly assays (75), and their presence in vertebrate HAT complexes suggests that they may assist in HAT activity in higher eukaryotes as well (28).

NPM, NASP, AND THE REMODELING OF PATERNAL CHROMATIN AFTER FERTILIZATION

During spermiogenesis, paternal chromatin undergoes extensive remodeling and condensation, resulting in a highly compact and transcriptionally silent conformation (for extensive reviews on this topic, see refs. 36, 80. As sperm head size affects both sperm motility and function (81); proper genome compaction within the sperm head is essential for fertilization (82). In some organisms, a key component in this process includes the replacement of histones by highly specialized sperm nuclear basic proteins (SNBPs), a group consisting of protamines (83), protamine-like proteins (83), and sperm-specific variants (84). Replacement of histones by SNBPs is thought to occur during final postmeiotic phases of spermatogenesis and results in a 10-fold compaction of paternal chromatin (85). Shortly following fertilization, sperm chromatin must be decondensed and reorganized by egg-supplied proteins. This remodeling of paternal chromatin allows for the formation of the male pronucleus within the egg and subsequently allows for proper zygote fusion

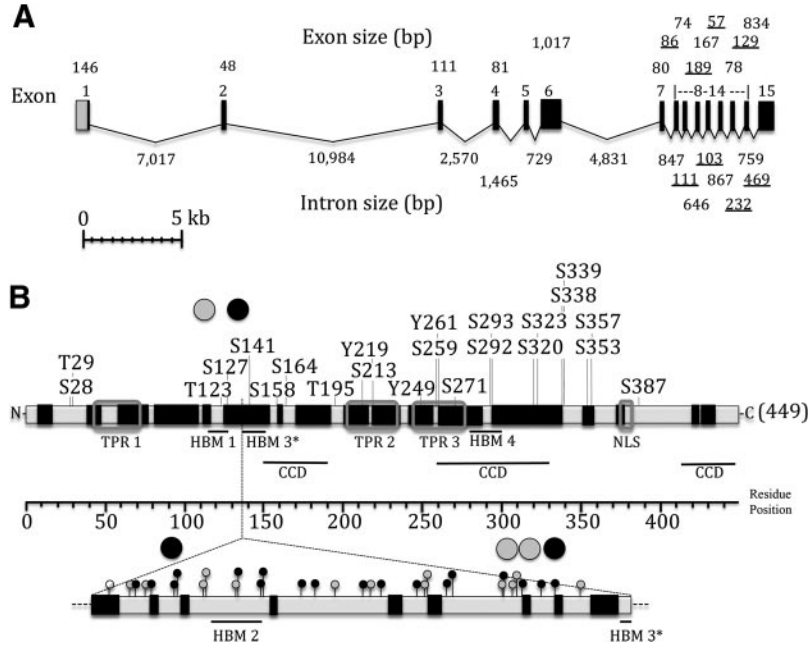

Figure 7. *A*) Schematic representation of the exon and intron organization of the *H. sapiens* NASP gene (NCBI GeneID: 4678). For a detailed description, see Figure 3*A*. Length S in base pairs of every other intron and exon is underlined for clarity in order to distinguish one intron/exon from another. *B*) Schematic diagram of *H. sapiens* NASP (UniProtKB: P49321) displaying the predicted secondary structure, selection of functional motifs and residues, and the sites of phosphorylation. Solid black boxes represent α-helical structure predicted by Phyre 2.0 server (93). Outlined in shaded boxes: TPRs, motifs implicated in protein-protein interactions, and NLS, which targets NASP for nuclear import. Segment of tNASP that is not present in sNASP due to alternatively splicing is bordered within the dashed lines. Solid black lines indicate the location of putative histone-binding motifs (HBMs), asterisk indicates that a protion of this HBM 3 motif is only present in the tNASP isoform. Regions labeled with a solid black line are the predicted CCDs, involved in protein stability and protein-protein interactions. The residues that are predicted to be phosphorylated according to the NetPhos 2.0 server (125), and those experimentally determined (126), are labeled as in Fig. 3*B*, with large solid black circles for phosphorylated serine residues, large shaded circles for phosphorylated threonine residues, and large open circles for phosphorylated tyrosine residues. Phosphorylation sites present only in tNASP are not numbered for clarity.

and development. Members of the NPM and NASP families of chaperone proteins are known to play a key role during paternal chromatin remodeling (71, 86). However, in mammals, it still remains unclear as to NPM2 and NPM3's exact roles within the egg.

Similarly to NPM2, NASP is very abundant in *X. laevis* eggs, where it was initially discovered and named N1/N2 (63). In contrast to NPM2, which stores H2A/H2B, NASP stores a soluble; nonchromatin bound pool of H3/H4 in *Xenopus*. Although NASP also serves as a storage for H3/H4 in somatic cells (~1% of the total H3/H4 in the cell; ref. 25) in the oocytes of some species, this amount can increase up to 100% larger than that of chromatin-bound H3/H4 (26).

NASP has been shown to be essential for early mouse embryonic development (40), and its expression levels have been determined to be elevated in embryonic stem cells (ESCs) and decreased on cell differentiation (68, 69). This is related to its role in H3/H4 storage and is responsible for maintaining a soluble H3/H4 pool to assist in the rapid chromatin remodeling that occurs after fertilization (1, 25, 26), seen in female development in *Caenorhabditis elegans* (63).

In addition to its presence in the egg, NASP has also been intriguingly found in sperm (14, 65). The occurrence of NASP in sperm and its potential involvement in fertilization deserves more extensive characterization. In male germ cells, tNASP is localized in the same regions as tightly packed chromatin, histones, and protamines; adjacent to the Golgi apparatus and nucleus of primary spermatocytes, in the nucleus acrosome and subacrosomal regions of rounds spermatids, and in the periacrosomal region of mature spermatozoa (64). In later stages of spermatogenesis, it is found primarily in the nucleus of later spermatids, testicular spermatozoa, and ejaculated spermatozoa, with traces remaining in the acrosome (64). While the full extent of NASP's role in sperm remains to be elucidated and the function associated with sNASP in spermatogenic cells (73) has yet to be defined, it is known that tNASP is responsible for the exchange of the mammalian testes-specific H1t in spermatogenic cells (65). Heat-shock protein 90 kDa (Hsp90) and tNASP form stable complexes in the cytoplasm, whereupon binding of H1t, the Hsp90 ATPase activity is stimulated (65). Through a yet to be determined mechanism, this ATPase activity induces the release of tNASP-H1t from Hsp90, and the tNASP-H1t complexes are subsequently translocated to the nucleus, where the histone is released for binding to chromatin (65).

STRUCTURAL AND CONFORMATIONAL FEATURES

NPM family

Crystallographic structures of the N-terminal domains have been determined for NPM1 in *Xenopus* (87) and humans (88), NPM2 in *X. laevis* (55), and humans (89), and nucleoplasmin-like proteins (NLPs) in *Drosophila melanogaster* (56). Each of these proteins comprises a protease-resistant N-terminal core and a highly disordered C-terminal tail (Fig. 1). In all instances, the N-terminal core has an 8-stranded, β-barrel topology that is believed to be responsible for pentamer oligomerization of its monomer subunits (refs. 55, 56 and Fig. 1*C, D*). Interestingly, and despite the predicted structural tertiary structure similarity of the N-terminal region of all the NPM members (ref. 86 and Fig. 1*C, D*), mammalian NPM3 appears to lack the ability to form homopentameric structures of its own and can only form heterologous oligomeric structures with NPM1 (37). A highly disordered C-terminal tail of NPMs has been suggested to play a key role in ligand binding (53–55, 89), and each member contains 2–3 conserved acidic tracts consisting mainly of glutamic acid (Fig. 1*A, B*). A short A1 acid tract presents within the N-terminal core is thought to be responsible for NPM oligomerization and chaperone activity (87, 90); however, it is not present in human NPM2, where the A1 loop contains only one acidic residue (Figs. 1*A* and 3*A*). A nuclear localization signal (NLS) is present within the C-terminal region of all NPMs (50), with NPM1 containing an additional nuclear export signal (NES) and an NLS.

There are many potential sites of post-translational modifications (PTMs) within the NPM and NASP family of chaperones, including methylation, glycosylation, N-myristoylation, ubiquitination, and acetylation. However, the most prominent and functionally significant PTM is phosphorylation. NPM2 activity is strongly correlated with its level of phosphorylation (91). During oocyte maturation, *X. laevis* NPM2 becomes hyperphosphorylated until the midblastula transition stage (92). Mass spectrometry analysis of *Xenopus* egg NPM2 and computational prediction analysis has identified phosphorylated residues within the N-terminal core and C-terminal tail (ref. 91 and Fig. 3*B*). The activation of NPM through hyperphosphorylation and its subsequent ability to interact with H2A/H2B dimers and sperm chromatin has previously been mimicked by substituting phosphorylatable residues with aspartic acid (91).

NASP

The two main human NASP isoforms differ considerably in size; tNASP is a protein of 788 aa, compared to sNASP, a protein of 449 aa. There is a 339-aa region present in tNASP that missing in sNASP due to alternative splicing of the mRNA transcript. Both isoforms are highly acidic, with a 24–25% glutamic or aspartic acid content, concentrated in an acidic stretch in the NH_2-terminal region (Figs. 4*A* and 5*A*). Secondary structure analysis of NASP and its homologs computationally predicts structures with ~50–65% α-helical content and 35–50% intrinsically disordered regions (33, 93), in agreement with the experimentally determined data obtained for human sNASP using circular

dichroism (33). NASP and its homologs possess several common features, including multiple TPRs, a region of coiled-coil domains (CCDs) and an NLS. TPRs are structural motifs involved in protein-protein interactions in multiprotein complexes, and could be possible sites of interaction with histones and other sNASP binding partners. At least 3 TPR motifs are present in human tNASP (residues 43–76, 542–575, and 584–617) and sNASP (residues 43–76, 203–236, and 245–278) (Figs. 4*A*, 5A, and 7*B*). CCDs are regions of α-helical structure that have been implicated in overall tertiary structural stability and as regions of protein-protein interactions, as exemplified by the interaction of intermediate filaments and nuclear lamins with other molecules. Although there is some variation based on the sequence prediction algorithm used with regards to the exact residues composing the CCD of human sNASP, the consensus indicates at least one likely CCD at approximately residues 261–315 of sNASP with a 99% probability according to MARFOIL 1.0 (94) and Phyre 2 Protein Fold Recognition Server (93). Park and Luger (95) have previously shown that the CCD region of NAP1 was responsible for its dimerization, and Wang and colleagues (74) have postulated that the CCD region of sNASP could also be the region responsible for its dimerization. However, there is also leucine zipper-like domain just to the NH_2-terminal of this region (residues 233–254) that could also be a region responsible for the dimerization of sNASP. Sedimentation velocity and equilibrium experiments by analytical ultracentrifugation and size-exclusion chromatography indicate that sNASP exists as a dimer in solution (18, 33) under physiological conditions. Putative histone-binding motifs have been characterized in previous studies of tNASP (residues 116–127, 211–244, and 469–512; ref. 96) and sNASP (residues 116–127, 138–172, and 280–300; ref. 33).

There are numerous experimentally determined and computationally predicted sites of phosphorylation on tNASP and SNASP (ref. 97 and Fig. 7*B*), and while their exact function is not known, experimental high-throughput proteomic evidence suggests that at least 35% of these phosphorylation sites are phosphorylated during DNA damage by ataxia telangiectasia mutated (ATM)/ataxia telangiectasia and Rad3 related (ATR), DNA-dependent protein kinase (DNA-PK), and other potential kinases (98, 99). Several of these phosphorylation sites are predicted to lie within the TPR motifs, the putative histone-binding domains and two of the CCD domains, shared by the s- and tNASP isoforms (97). Phosphorylation of these residues could directly affect the ability of sNASP and tNASP to bind histones and other binding partners, thereby altering their chaperone activity. The proteomic identification of NASP-binding partners in HeLa cells and yeast (68) includes the Ku protein 70 kDa (Ku70)/Ku protein 80 kDa (Ku80) protein subunits [X-ray repair complementing defective repair in Chinese hamster cells 6 and 5 (*XRCC6* and *XRCC5*) in Fig. 4] involved in the nonhomologous end joining (NHEJ)-DNA repair (68), the presence of the NASP homologue, Hif1p, at yeast DSBs (61, 79), and NASP's hyperphosphorylation during DNA damage, suggest that NASP may play a vital role in DNA repair.

HISTONE STORAGE AND CHAPERONE ACTIVITIES

NPMs

In mammals, NPM2 has been proposed to play a role in the formation of nucleolus-like bodies (NLBs) within the egg, a process involving the lysine-rich, C-terminal motif of NPM (100). Although the exact roles of these NLBs are still not well understood, they have recently been shown to be essential for early embryonic development (101). Mammalian NPM2 is also believed to play a role in early embryogenesis shortly following fertilization (102). When NPM2 and NLBs were removed from mature oocytes, proper chromatin decondensation was significantly hindered (35). Interestingly, the same study found that H2A/H2B deposition onto paternal chromatin remained unaltered by the NPM2 removal process. This is in contrast with *Xenopus* NPM2 where this protein, in itself, is critical for histone H2A-H2B deposition and chromatin remodeling of the male chromatin after fertilization (52). This functional disparity between mammalian and *Xenopus* NPM2 invariably highlights a possible difference between NPM2's role among various species. Other studies have shown that NPM2 alone in mammals does not appear to be sufficient for sperm DNA decondensation after fertilization and may suggest that other NPM family members could be compensating in this group of organisms during early zygote development (35, 36).

Twenty years following the discovery of NPM2, the third member of the NPM family, NPM3, was isolated and characterized in mice (103). Similar to NPM1, NPM3 is ubiquitously expressed across various tissues (104), and although its exact roles are still being understood, NPM3 has been implicated in the regulation of NPM1 activity during ribosomal RNA genesis (38). NPM3 is also expressed within mammalian oocytes, and research suggests that this protein may be critical for proper sperm chromatin decondensation (36). A study looking at sperm chromatin decondensation in mammals targeted two oocyte proteins: NAP1 and NPM3 (81). Here, researchers determined that microinjection of NPM3 and NAP1 antisense oligonucleotides together, or NPM3 antisense oligonucleotides alone, in mouse oocytes abolished proper histone assembly (detected by immunohistone presence) and subsequently hindered the ability of paternal chromatin to progress beyond the dispersed state. This may suggest that NPM3 is also acting as a key histone chaperone protein within the mammalian oocyte and highlights the need for future experiments in order to determine the exact role of NPM family members present within the egg of these organisms.

At the molecular level, information available on the binding of histones to nucleoplasmin has been mainly obtained with *X. laevis* NPM2. Despite the acidic nature of this molecule and the basic charge of its interacting histone partners, it has been shown that hydrophobic interactions play a critical role more so than that of the electrostatic component (91, 105). Interestingly, functional phosphorylation activation of NPM2 (see Fig. 3*B*) enhances the binding affinity for histones. However, such an increase appears to be related to changes in the quaternary pentameric rearrangement of the molecule rather than in the negative charge increase associated with phosphorylation (106). In its pentameric form, *Xenopus* NPM2 can bind up to 5 molecules of H2A/H2B and linker histones with decreasing affinity as the number of bound histones increases (0.1 to 200 nM K_d for H5 and 65 nm to 1 μM for H2A/H2B; ref. 105). The differential affinity of nucleoplasmin for histone H5 (linker histones) and H2A/H2B may facilitate its dual functional role as a storage histone chaperone and as a chromatin remodeler (105).

NASP

Histone production is tightly controlled by the regulation of mRNA processing, gene transcription, and protein degradation pathways. It was believed that histones were synthesized at levels to cover what was required during specific cell stages for chromatin assembly (107). However, situations exist in which histones are rapidly required as a result of unforeseen events, and recent evidence suggests that in somatic cells, a contingency cytoplasmic and nuclear reservoir of soluble H3/H4 comprising ~1% of the total H3/H4 content is maintained. These soluble H3/H4 levels can be adjusted by altering the levels of sNASP produced in the cell (25). Somatic NASP is capable of compensating for altered levels Asf1 and could even compensate for H3/H4 overproduction by marking excess H3/H4 for protein degradation through chaperone-mediated autophagy (CMA) by the chaperone's capacity to control the activity of its cochaperone's Hsp90 and heat shock cognate 70 kDa (Hsc70) (25). Having precise control of H3/H4 levels allows sNASP to compensate for immediate deficiencies in H3/H4 and provide stability to the genome under conditions of stress that could result in further cell cycle arrest or damage (25) and by increasing the reservoir of H3/H4 to allow for continued cell growth (25). It has been shown by these researchers that, by varying the levels of sNASP, somatic cells are able to alter the levels of H3/H4 available. The level of the NAS H3/H4 reservoir can be reduced by as little as 10% of the normal levels present or doubled (25). However, the extent to which somatic cells are able to modify levels of sNASP on their own to compensate for these H3/H4 production alterations is not known.

Both tNASP and sNASP are present in chromatin-remodeling complexes associated with the deposition of H3/H4, and their ability to bind H3/H4 *in vivo* has been well documented (18, 25, 28). While tNASP had initially been described as a linker histone chaperone *in vivo* (14, 61, 65, 70), recent evidence suggests it also has a chaperone role for newly synthesized H3/H4 (18). Somatic NASP's role as a linker histone chaperone has been demonstrated *in vivo* and *in vitro* (18, 25, 33, 34, 65, 67, 73–75). *In vitro* sNASP binds to linker histones with high affinity (74, 75), and chromatin assembly assays have demonstrated its ability to deposit linker histones onto chromatin fractions depleted of H1 and to restore the histone H1-dependent folding, which is characteristic of native chromatin (33).

Somatic NASP was first described as a member of certain histone chaperone-containing complexes encompassing Asf1, CAF-1, HIRA, and histone H3/H4 in somatic cells (18, 25, 28, 68). It was later demonstrated to have H3/H4 chaperone activity *in vitro* (34, 74, 75) and *in vivo* (34, 74) by assisting in the assembly of tetrasomes and nucleosomes, a H3/H4 chaperone activity, also shared by its homologs Sim3 (17) and Hif1p (61, 74, 75). Human sNASP has been found to effectively promote the assembly of H3/H4 tetrasomes (34) and nucleosomes consisting of different H3 variants, including H3.1, H3.2, H3.3, and the centromere-specific H3 variant, CENP-A, with the exception of testes-specific H3 variant (H3T; ref. 34). Mutational analyses have shown that the Met-71 residue of H3T is responsible for this inefficient assembly of nucleosomes (34). The NH_2-terminal region to central region of sNASP (residues 26–325) has been shown to be essential for sNASP's interaction with linker and core histones and its function in nucleosome assembly (34, 74).

Surface plasmon resonance (SPR) biosensor analysis used to measure the binding kinetics and affinities of histones with NASP determined the dissociation constant, K_d, of full-length sNASP interacting with H1 and with H3/H4 to be 237 and 13.8 nM, respectively (75). These obtained values are within the range of K_d values of many histone chaperone-histone interactions, but almost in reverse to that of NPM2, binding to linker histones more tightly than to H2A/H2B (see previous section). To further elucidate the specificity of sNASP's binding affinity for H3, H4, and H1, SPR analysis was conducted on a series of deletion constructs. On the basis of these results, it appears that distinct and separate sNASP domains interact with linker histones and core histones. In particular, the NH_2-terminal region to central region of the protein (residues 26–325) is essential for sNASP's interaction with both linker and core histones and its function as a nucleosome assembly factor (34, 74). The acidic polyglutamic region in the NH_2 terminus (residues 116–172) of sNASP is required for H1 interaction but not for core histone interaction (74). It appears that in this case, the electrostatic interaction between the acidic region in the NH_2-terminal portion of sNASP and the basic, lysine- and arginine-rich, NH_2-terminal and COOH-terminal intrinsically disordered tails of H1 are essential for the interaction of H1 with sNASP (33), whereas

the region around the COOH-terminal TPR motifs (residues 196–281) is essential for H3/H4 interaction (74). *In situ* experiments with size-exclusion chromatography and immunoblot detection indicate that sNASP forms complexes with either H3/H4 or H1 but not with both at the same time (74).

Although the sedimentation velocity and equilibrium experiments by analytical ultracentrifugation indicate that sNASP exists as a dimer in solution (33), the binding stoichiometry of sNASP with H3/H4 and histone H1 is more complex and difficult to elucidate. Although there is association between sNASP and H3/H4, it could not be determined whether sNASP was associating with H3/H4 dimers or H3/H4 tetramers (18).

RELATIONSHIP OF NPM1 AND NASP TO CANCER

As cell growth, proliferation, and differentiation are critical to cancer cells and their ability to spread, it is not surprising that NPMs and NASP play key roles in oncogenesis. In this regard, NPM1 is the most extensively studied member of the NPM family, largely due to its role in tumorigenesis (108) and hematopoietic malignancies (109). NPM1 has strong links to the murine double minute-2 (MDM2)/p53 tumor suppressor pathway and has been shown to play an important role in p53 stability and transcriptional activation (110). Strikingly, 55 different mutations in exon 12 (111) of NPM1 have been found in 35% of all adult acute myeloid leukemia (AML) cases (109), suggesting that the altered NPM protein may have a direct connection to AML progression. Characteristic of these mutations is the addition of a NES motif and an alteration in the NLS motif in the C-terminal region (112). Mutations leading to the disrupted NLS, a motif responsible for shuttling the protein from the cytoplasm into the nucleoplasm, results in irregular NPM1 build-up in the cytoplasm (112).

While mutations in NPM1 occur in a large percentage of AML cases, there appears to be an absence of NPM1 gene mutations found in other forms of common solid cancers, including cancer of the lungs, liver, breast, colon, and gastric carcinomas (113). Instead, overexpression of NPM1 has been noted in solid tumors from various histological origins (108). In both instances, the deregulation of NPM1 contributes to tumorigenesis, highlighting this protein's importance in a wide variety of mechanisms within the cell.

One of the distinctive features of cancer is genome instability caused by DNA replication stress (114). The full extent of NASP's relationship in oncogenesis is only now being understood, as significant changes in its expression levels have been observed in a variety of cancers (66, 72, 115), including prostate (69) and ovarian cancer (116). For example, high-throughput microarray data suggest that NASP may play a significant role in the development and progression of various cancer cells, signifying its use as a potential biomarker or prognosis tool. For example, NASP is expressed in highly elevated levels in ovarian cancer tissues compared to normal ovarian tissue and serous cystadenomas (116). The only other statistically significant alteration in expression levels concomitant with those of NASP was for meiotic recombination 11 homolog (MRE11), another protein critical for DNA repair, which was expressed at much lower levels (116). In fact, only NASP and MRE11 proved statistically significant in predicting the development of cancerous cell growth and terminal fate in these types of cancer (116).

It is possible that a change in NASP levels, and therefore a subsequent change in the amount in soluble H3/H4, could result in reduced genome integrity, chromatin stability, tumorigenesis, and replication stress (25). Examples of this have been demonstrated in fission yeast, where perturbed core histone levels resulted in errors in mitosis (117), DNA replication (118), and replication fork stalling (119).

MOLECULAR EVOLUTION OF NASP AND NPM

The NASP/NMP chromatin remodeling similarities during early fertilization (binding to histones and protamines) are not mirrored by their protein primary structures beyond the common occurrence of stretches of acidic amino acids, which may potentially be involved in electrostatic interactions. Molecular evolutionary analyses indicate that NASP and NPM constitute different protein lineages (120). Further support to this suggestion is provided by the basal position occupied by NPM in the phylogeny of NASP, as well as by the root position of NASP in the phylogeny of the NPM family shown in **Fig. 8*A***.

Nevertheless, the long-term evolution of NASP seems to have shared some common features with those displayed by members of the NPM family, most notably the differentiation of somatic and germinal NASP isoforms. Although this holds true in terms of functional diversification, the evolutionary pathways followed by each chaperone seem to be quite different. For one, our phylogenetic analyses suggest that NASP isoforms have most likely arisen polyphyletically across different vertebrate lineages (mostly eutherian mammals), leading to a closer relationship among the different NASP isoforms within taxa (Fig. 8*A*). This process resembles that followed by other chromatin-associated proteins such as H2A.X and noncentromeric H3 variants (121). In addition, we have already reported that NPM family members (120) in contraposition to NASP display a clustering pattern based on their type (instead on the species to which they belong) in the phylogenies (Fig. 8*A*). Such differentiation pattern reveals a process of functional evolution, which results from well-defined and strong selective constraints operating across different family members (120).

Given the nature of the interactions between NASP/

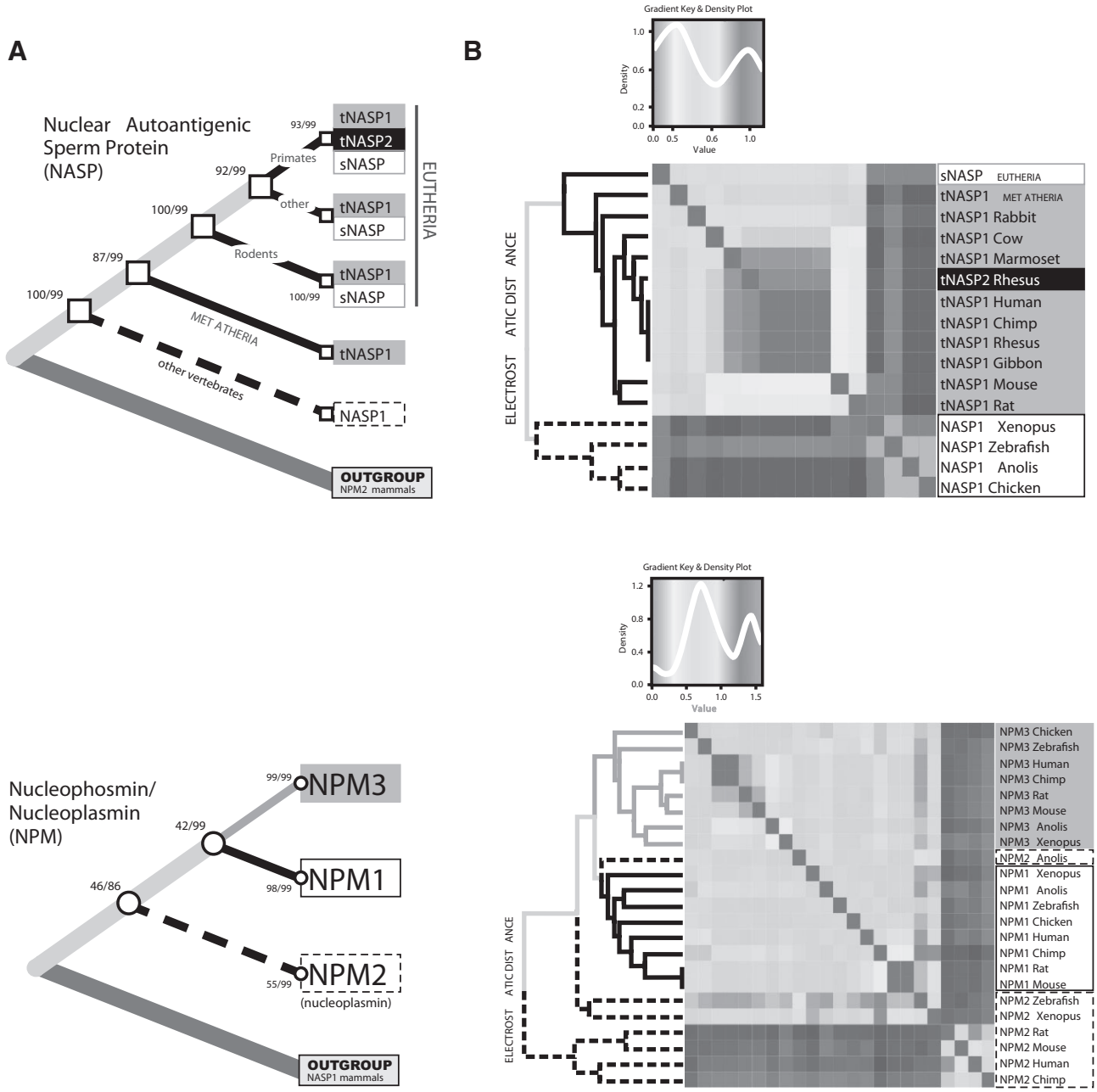

Figure 8. Evolution of NASP and NPM histone chaperones. *A*) Simplified maximum-likelihood phylogenetic tree topologies depicting the evolution of NASP and NPM protein lineages across vertebrates. Different NASP isoforms are more closely related within taxa ("other" accounts for other noneutherian mammals), while NPM family members display a clear differentiation among paralogs based on their function. Numbers at internal nodes indicate bootstrap and internal branch-test significance values (1000 replicates). *B*) Electrostatic distances among members of NASP and NPM families across vertebrates. Distances are calculated using webPIPSA (127) comparing the electrostatic potentials of different NASP and NPM members, represented in a gradient-coded matrix (heat map). Gradient code and number of comparisons for each distance interval are indicated in the gradient key and density plot near epograms. Tree along the side of the image assembles the proteins into groups with similar electrostatic potentials (epogram), mirroring the functional differentiation within NASP (sNASP and tNASP) and NPM (NPM1, NPM2, and NPM3) chaperone families.

NPM and histones, it seems reasonable to assume that both chaperones are subject to strong selective constraints in order to preserve an appropriate (acidic) charge, necessary to correctly bind histones. The analysis of the electrostatic properties shown in Fig. 8*B* suggests that this is, indeed, the case. The agreement between the protein functional specialization depicted by the epograms (Fig. 8*B*) and the phylogenetic relationships among NASP and NPM types (Fig. 8*A*) suggests that electrostatic potentials constitute critical targets for selection during the evolution of these chaperones. Thus, it appears that modifications in key residues affecting the overall electrostatic potential could constitute the mechanistic basis for the functional differentiation of NASP and NPM types, as it has been previously reported for the case of histones (122).

CONCLUSIONS

NPM2 and NASP (N1/N2) were both originally described to be massively present in the eggs of *X. laevis* bound to core histones H2A/H2B and H3/H4, respectively (62). N1/N2 was subsequently rediscovered under a completely different name (NASP; refs. 59, 123) as a linker histone H1 chaperone. Both chaperones bind with substantially different affinities for core and linker histones, likely as a result of a dual chaperone and chromatin-remodeling activity. Whereas the contribution of NPM2 to the paternal chromatin remodeling that takes place after fertilization has been extensively characterized (see ref. 111; for review, see refs. 84, 91), the role of NASP in such process remains completely unexplored. This is also mirrored by the comparatively low amount of structural information (nonexistent for tertiary and quaternary structures) available for NASP when compared to NPM (ref. 111 and references cited therein). It is also not clear how the H1 and H3/H4 chaperone activities of sNASP and tNASP are precisely mediated, but in somatic cells, sNASP cannot bind both H3/H4 and H1 (74). The presence of NASP in sperm (64, 65) is also intriguing and deserves further examination. These functional and structural aspects of NASP biology should be forthcoming and may provide valuable information. Also, as discussed above (see Figs. 2 and 6), these two histone chaperones have a plethora of interacting partners, through which they are involved in many different aspects of nuclear metabolism. The potential involvement of NASP in processes, such as DNA replication, DNA recombination, and repair, and cell proliferation requires further attention. Finally, a close examination of the evolutionary mechanisms underlying the functional specialization found among NASP family members will help integrate their long-term evolution in the context of chromatin structure and dynamics. FJ

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