New insights into the nucleophosmin/nucleoplasmin family of nuclear chaperones

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Summary

Basic proteins and nucleic acids are assembled into complexes in a reaction that must be facilitated by nuclear chaperones in order to prevent protein aggregation and formation of non-specific nucleoprotein complexes. The nucleophosmin/nucleoplasmin (NPM) family of chaperones [NPM1 (nucleophosmin), NPM2 (nucleoplasmin) and NPM3] have diverse functions in the cell and are ubiquitously represented throughout the animal kingdom. The importance of this family in cellular processes such as chromatin remodeling, genome stability, ribosome biogenesis, DNA duplication and transcriptional regulation has led to the rapid growth of information available on their structure and function. The present review covers different aspects related to the structure, evolution and function of the NPM family. Emphasis is placed on the long-term evolutionary mechanisms leading to the functional diversification of the family members, their role as chaperones (particularly as it pertains to their ability to aid in the reprogramming of chromatin), and the importance of NPM2 as an essential component of the amphibian chromatin remodeling machinery during fertilization and early embryonic development.

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Abbreviations: MBT, mid blastula transition; NES, nuclear export signal; NLS, nuclear localization signals; NoLS, nucleolar localization signal; dNPL, *Drosophila* nucleoplasmin-like protein; NPM, nucleophosmin/nucleoplasmin family; NCP, nucleosome core particle; PARP-1, Poly(ADP-ribose) polymerase1; SCNT, somatic cell nuclear transfer; SNBPs, sperm nuclear basic proteins; SPs, sperm-specific proteins.

Introduction

Charged molecules within the cell, such as nucleic acids or proteins, must have their charges shielded to prevent aggregation, which can occur when opposing charges on molecules improperly interact. This is the case for nucleic acids and their interactions with histones in which incorrect binding may result in aggregation instead of the assembly of nucleosome core particles (NCPs). NCPs are the complex of DNA and histones in which approximately 146 base pairs of DNA are wrapped around an octamer of core histones (two H2A–H2B heterodimers and an H3–H4 tetramer). The addition of histone H1, which binds to the linker DNA between the NCPs, protects an additional 20 base pairs of DNA creating the chromatosome.[1,2] Nuclear chaperones are needed for the proper assembly of nucleosomes and the attainment of proper higher order chromatin structures. The nucleophosmin/nucleoplasmin (NPM) family of nuclear chaperones has members found throughout the animal kingdom, which can be subdivided into the following four groups based on their protein sequences: NPM1, NPM2, NPM3 and invertebrate NPM proteins[3,4] (see Table 1).

Nucleoplasmin (NPM2) was first isolated from eggs and oocytes of the African clawed frog, *Xenopus laevis*,[5] in which it is the most-abundant nuclear protein.[6] Nucleoplasmin binds to histones and mediates the assembly of nucleosomes from DNA and histone proteins.[5,7] It also binds sperm nuclear basic proteins (SNBPs)[8–10] in order to facilitate the decondensation and remodeling of paternal chromatin following fertilization. Nucleophosmin (NPM1) was first identified as a phosphoprotein that was highly expressed in the nucleolus.[11,12] NPM1 was initially thought to be important for the assembly of ribosomes and has since been found to have roles in many important cellular processes (see Table 1). NPM1 is the most studied of the NPM family members, largely due to the fact that it is often upregulated in tumors and is a frequent target of genetic alterations in cancers (recently reviewed in Ref. 13). In this review, we will focus specifically on the role of NPM1 as a histone chaperone. The least amount of data is

Table 1. NPM family members

Member	Also known as*	Location	Properties and functions
Nucleophosmin/nucleoplasmin member 1 (NPM1)	Nucleophosmin, also called B23[11,12] or numatrin[68] in mammals and NO38 in amphibians[69]	Mainly nucleolar; wide tissue distribution[70,71]	Roles in several cellular processes, including: ribosome biogenesis,[70,72–74] nucleocytoplasmic transport,[75–78] centrosome duplication,[58,59] embryonic development and genome stability,[79] DNA duplication,[80–82] transcriptional regulation[53,83] histone chaperoning,[52,53] binding and folding of denatured proteins[84,85] and nucleic acid binding[86–88]
Nucleophosmin/nucleoplasmin member 2 (NPM2)	Nucleoplasmin in amphibians[7]	Nuclear; only found in eggs and oocytes[5,6]	Binds histones and promotes chromatin assembly[5,23] paternal chromatin decondensation[46,89]
Nucleophosmin/nucleoplasmin member 3 (NPM3)	NO29 in amphibians[15]	Mainly nucleolar;[16] wide tissue distribution[3]	Ribosomal RNA biogenesis[16] and potentially paternal chromatin decondensation in mammals[17]

*The nomenclature for this family has been complicated by different names given to the proteins by different groups. For this review the abbreviations NPM1, NPM2 and NPM3 will be used.

available for NPM3,[14,15] which is the most recently discovered family member. NPM3 has been implicated in the regulation of NPM1 in ribosomal RNA genesis[16] and, in addition, its expression in mammalian oocytes has been correlated with paternal chromatin decondensation,[17] similar to NPM2 in lower vertebrates. Invertebrates also have NPM-like family members, the best characterized of which is the *Drosophila* nucleoplasmin-like protein (dNPL).[18] Although dNPL is able to bind histones and decondense sperm chromatin, this nucleoplasmin homologue is different from the archetypical nucleoplasmin in *Xenopus* in that it is dependent on ATP to assemble nucleosomes.[19,20]

In this review, the information on NPM gene and protein structures and evolution is comprehensively presented and discussed with respect to the chaperone role fulfilled by the members of this family, particularly as it pertains to their contribution to chromatin remodeling and reprogramming.

A family of evolutionarily related proteins

Gene and protein structure

NPM1 members contain twelve exons in human and rat, and eleven exons in mouse. The human NPM1 gene can be transcribed as three variants among which isoform 1 is the predominant type and results in the longest transcript. In contrast, the shorter isoforms 2 and 3 lack an in-frame exon (exon VIII) and utilize an alternate 3′ exon (exon X), respectively. Rat, NPM1 can also be transcribed as one of two alternative mRNAs that result in two different proteins termed B23.1 and B23.2.[21] NPM2 members have eight exons while NPM3 members have only five exons (with the exception of rat, which has an additional exon) (Fig. 1). *Drosophila* dNLP, taken here as a representative of the invertebrate NPM-like lineage, has a coding region composed of only three exons. The relative number of exons is generally proportional to the overall protein length of the corresponding NPM type and, although the intron sizes exhibit significant heterogeneity, the sizes of the exons are relatively conserved within each type.

The evolutionarily related members of the NPM family share similarities not only in their primary amino acid sequences, but also in their domain organization and tertiary structure (Fig. 2). An N-terminal protease-resistant core, which is the most-conserved domain between these proteins and, in most cases, includes a short A1 acid tract, is responsible for oligomerization and chaperone activity. A second region, the C-terminal tail domain, contains up to two additional acids tracts (A2 and A3) and has variable length and functional motifs between the family members.[22] In NPM2, the first of the C-terminal acid tracts (A2) is the longest whereas in NPM1 this first tract is relatively short and followed by an additional region that is not conserved in the NPM2 or NPM3 sequences (Fig. 2). For NPM1, the A3 region is the longest of the three acid tracts, similar in length to that of A2 in NPM2. All three have classic bipartite nuclear localization signals (NLS) with the consensus sequence $KRN_{10}KKK$, where N accounts for any amino acid.[23] However, while NPM3 terminates immediately following this NLS, NPM2 extends into the last acid tract, which is absent in NPM3. Additional motifs which are exclusively found in NPM1 include the putative nuclear export signal (NES) and the extension at the C-terminal end containing the putative nucleolar localization signal (NoLS) and the domain for nucleic acid binding and RNA cleavage.[24]

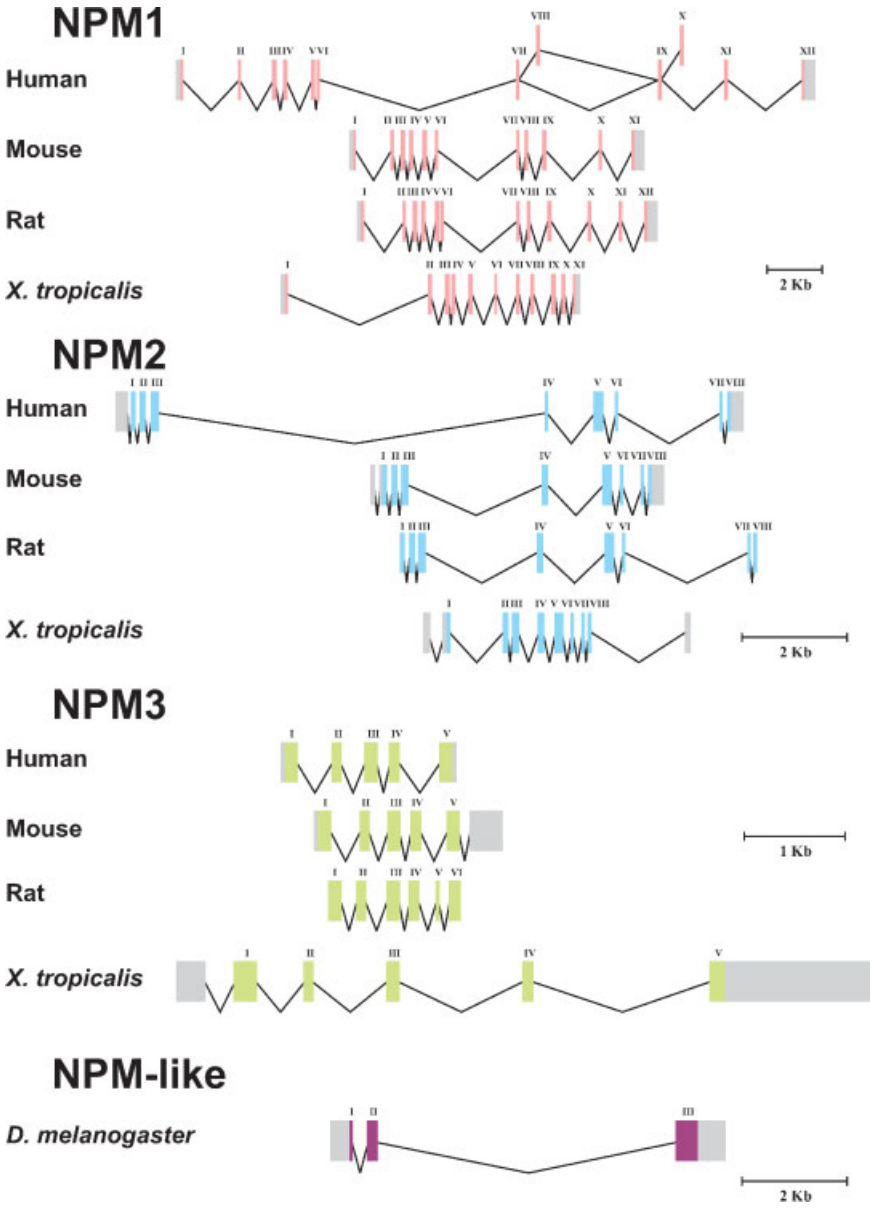

Figure 1. The intron and exon organization of NPM genes. Exons coding for translated regions of NPM1, NPM2 and NPM3 NPM-like proteins are schematically represented by pink, blue, green and purple boxes, respectively. Exons coding for untranslated regions are depicted by grey boxes. Introns are indicated by black lines. The *Xenopus tropicalis* NPM1, NPM2 and NPM3 sequences were found on the JGI *Xenopus tropicalis* v4.1 database and had assigned names of fgenesh1_kg.C_scaffold_24000023, fgenesh1_kg.C_scaffold_34000011 and fgenesh1_kg.C_scaffold_238000002 respectively. In the case of mammals, the accession numbers for the sequences used are as follows: NPM1 (human, NC_000005.8; mouse, NC_000077.3; rat, NC_005109), NPM2 (human, NC_000008.9; mouse, NT_039606; rat, NW_047454), NPM3 (human, NC_000010; mouse, NC_000085; rat, NW_047567); NPM-like (*Drosophila*, NT_033777.2).

It is worth noting that the human, mouse and rat NPM2 proteins do not contain an A1 acid tract in the N-terminal core region. It has been found that mutation of only four of the acidic amino acid residues of the A1 tract to glutamine residues drastically reduces the sperm decondensing activity of *Xenopus* nucleoplasmin and the ability of this mutant chaperone to swell sperm nuclei.[25] This is very interesting considering that mouse NPM2 was found not to be essential for protamine removal and sperm decondensation in knock out experiments (discussed further below). In contrast, human

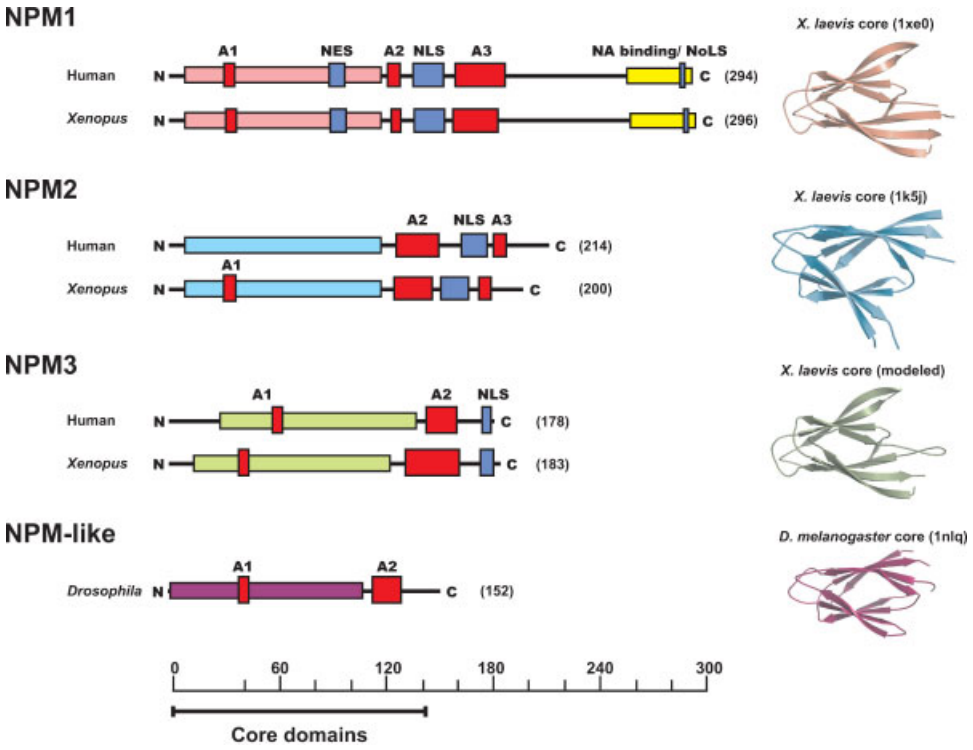

Figure 2. Selected NPM protein structures. Depictions of the protein domains on the left and the corresponding protein tertiary structures of the monomer core domains on the right. Acid tracts are shown in red. Localization signals are shown in blue (nuclear localization signal, NLS; nuclear export signal NES; and nucleolar localization signal NoLS). The core domains are shown in pink, blue, green and purple for NPM1, NPM2, NPM3 and NPM-like proteins respectively. NPM1 members also have a nucleic acid (NA) binding domain at the C terminus which is shown in yellow.

NPM3 protein, which does have an A1 tract in the N-terminal region, was shown to be required for decondensation of sperm chromatin.[17]

The tertiary structures of both the *Xenopus* NPM2 (nucleoplasmin)[26] and NPM1 (NO38)[27] N-terminal core domains have been characterized by X-ray crystallography, as has the *Drosophila* nucleoplasmin-like core domain.[28] All three have monomer subunits with eight-stranded β-barrel topologies (Fig. 2) which were confirmed to form pentameric complexes in the cases of nucleoplasmin and NO38.

Long-term evolution and functional diversification

The intron and exon organization of NPM genes described above (Fig. 1) supports the functional evolution and diversification of the NPM family. The phylogenetic analyses of the different NPM family members among different metazoan taxa reveal a clustering pattern by type, instead of by species (Fig. 3). The observed topologies revealed a process of functional evolution operating in NPMs which ultimately must have imposed well-defined and strong selective constraints on the family members, resulting in the appearance of the different NPM (NPM1–NPM3 and NPM-like) types.

The NPM1 and the invertebrate NPM-like lineages are both characterized by a monophyletic origin in the protein topologies, whereas NPM2 and NPM3 have a polyphyletic origin due to what appears to be independent differentiation in mammals with respect to the rest of the vertebrate groups (see Fig. 3). The NPM-like lineage from invertebrates shows a closer phylogenetic relationship with the NPM1 lineage, although within invertebrates the characteristic acidic tracts are not as conserved as within vertebrates in terms of their spatial arrangement and consensus sequences. The diversification and distribution of the potential phosphorylation sites in NPM proteins across these different groups of vertebrates is also consistent with the polyphyletic origin described for NPM2 and NPM3, and further supports their differentiation early in NPM evolution.

In terms of gene evolution, NPM coding sequences diverge extensively through silent substitutions, which in all cases are significantly greater than the observed nonsilent variation. This reveals a deviation from neutrality that suggests the presence of selection acting on specific residues.[4] Indeed, the triplets encoding the glutamic and aspartic acid residues in the acidic tracts show bias in terms of nucleotide frequencies

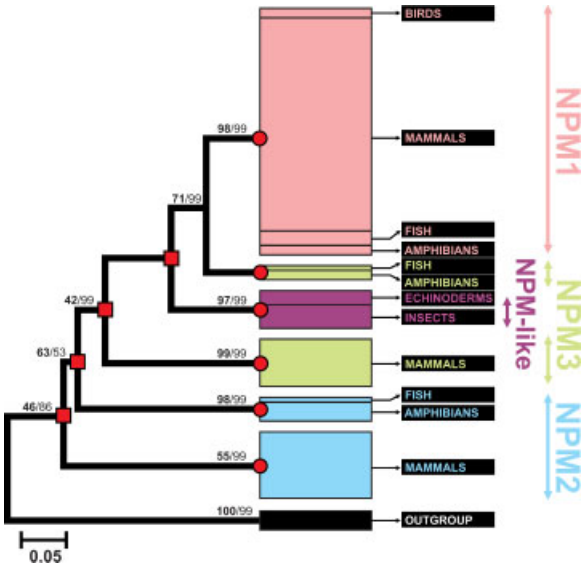

Figure 3. Schematic representation of the phylogenetic relationships among NPM proteins based on results obtained by.[4] The tree was reconstructed based on evolutionary protein *p*-distances using the pairwise deletion option. Numbers for interior nodes indicate bootstrap (boldface) and interior branch-test (normal) confidence values based on 1000 replications. The differentiation events leading to the appearance of the different NPM lineages are represented by red squares at the corresponding nodes in the tree, while diversification events are represented by red circles. The heights of the boxes in the right margin of the tree are proportional to the number of sequences analyzed for each taxa.

at the second codon positions, further supporting a departure from the neutral expectations and pointing towards a selection process favouring specific amino acids.[4]

It appears that the high frequencies presented by acidic residues at C-terminal regions (which are critical for the correct interactions of NPMs with other proteins) are maintained by selection, altering the nucleotide composition of these domains. Such a selection process for highly biased amino acid frequencies has been previously described in other genes such as the *tolA* gene from proteobacteria (maintaining high levels of alanine and lysine)[29] and the mammalian protamine 1 gene (high levels of arginine).[30]

Interestingly, the long-term evolution of NPM proteins shares some similarities with that of histones, for which NPM1 and NPM2 operate as chaperones. Both groups of proteins are under a strong purifying selection process at the protein primary structure level and diverge extensively through silent substitutions at the nucleotide level.[4,31] An intriguing possibility would involve the parallel evolution of these two groups of interacting proteins, which could have ultimately resulted in the functional diversification of the different NPM lineages. However, more functional and structural data are still needed before this hypothesis can be tested.

Overview of chaperone functionality

The archetypical Xenopus nucleoplasmin: sperm chromatin remodeling

Accumulated biochemical studies have shed light on the possible mechanisms of nucleoplasmin binding to sperm-specific proteins (SPs) and histones[22] (Fig. 4). A model has been put forward for *X. laevis* in which histones bind to the lateral face of the nucleoplasmin pentamer through stereo-specific interactions.[22] When hyperphosphorylated nucleoplasmin, with H2A–H2B dimers bound to the lateral face, comes into contact with the highly compacted sperm chromatin, the SPs subsequently dissociate and bind to the distal face of the pentamer through electrostatic interactions. This SP–nucleoplasmin interaction may cause a conformational change in the nucleoplasmin pentamer resulting in the dissociation of the bound H2A–H2B dimers. Such a dissociation occurs within the vicinity of the paternal DNA with H3–H4 tetramers, thus allowing nucleosomes to form.[22]

In the egg, chaperones other than nucleoplasmin are also present and aid in chromatin remodeling. In *Xenopus* oocytes, H2A–H2B and H3–H4 are found complexed with nucleoplasmin and N1/N2, respectively.[32,33] Both chaperones are able to separately reconstitute nucleosomes in vitro, but are more efficient when used together.[34] In addition to the better known role of NAP1 as an H2A–H2B chaperone, *Xenopus* NAP1 (xNAP1) was recently found to immunoprecipitate with the *Xenopus* early embryonic linker histone B4.[35] In the absence of xNAP1, nucleosomes were assembled on sperm chromatin. However, there was an excessive deposition of B4 and micrococcal nuclease digestion showed abnormal chromatosome formation. These observations support the notion that xNAP1 presumably plays a chaperone role for the proper deposition of B4 in *Xenopus* eggs during early development, leading to a correct chromatin structure configuration (Fig. 4).

A larger picture in which these histone chaperones (nucleoplasmin, N1/N2 and NAP1) act together to coordinate chromatin remodeling following fertilization is beginning to be unveiled (Fig. 4). Nucleoplasmin seems to be the most important of the three for the initial decondensation of the sperm chromatin. Due to the fact that histones H3 and H4 are already present in the paternal chromatin of *Xenopus*, N1/N2 would have less of a role to play in this initial step. Following the exchange of H2A–H2B dimers for SPs by nucleoplasmin, NAP1 adds B4 and the chromatin in the paternal pronucleus then resembles that of the maternal pronucleus. Following replication of the paternal DNA, N1/N2 would be needed to deposit H3–H4 tetramers before nucleoplasmin could add H2A–H2B dimers (Fig. 4). This notion suggests a role for nucleoplasmin which continues into early development. In

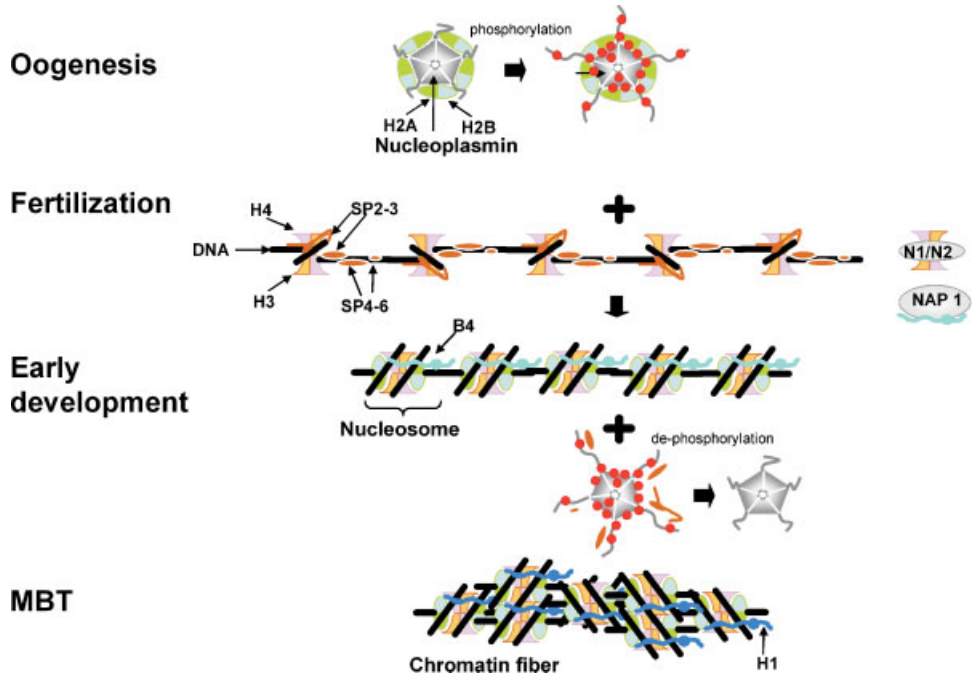

Figure 4. In the unfertilized egg, nucleoplasmin (grey pentamer) stores H2A–H2B dimers. During oocyte maturation, nucleoplasmin becomes phosphorylated, represented by red dots. The paternal chromatin in *Xenopus laevis* has sperm-specific proteins SP1–SP6 and H3–H4 tetramers. Nucleoplasmin removes the SPs from the paternal chromatin and supplies H2A–H2B dimers. The other chaperones, N1/N2 and NAP1, supply H3–H4 octamers and oocyte-specific linker histone B4, respectively. Nucleoplasmin remains phosphorylated until the mid-blastula transition (MBT).

support of this, phosphorylated nucleoplasmin is present until the mid blastula transition (MBT), after the first dozen divisions.[36] In addition, nucleoplasmin was also found to be present in the frog *Rana catesbeiana*, which contains only histones in the sperm and does not necessarily require nucleoplasmin for the initial step following decondensation, except in its capacity as a general decondensation factor.[37]

Why is there a need for different chaperone proteins than those found in somatic cells or, in the case of NAP, performance of a different task (chaperoning B4)? In *Xenopus*, transcription does not occur until after the MBT. Before this stage, rapid and synchronous rounds of replication occur approximately every half hour without increase in the size of the embryo. As no transcription occurs, all the required histones are stored maternally before fertilization. For this storage, and possibly to keep up with the replication machinery, the cell has embryo specific histone chaperones. In addition, embryos also contain specialized histones such as the linker histone B4 which has a longer, less basic C-terminal tail than somatic H1 and may bind less tightly to chromatin, thus allowing for better access by chromatin remodeling factors.[38] This would be important during early embryonic development when rapid rounds of replication are taking place. Nucleoplasmin becomes dephosphorylated at the MBT and, at this point, histone B4 is replaced by the conventional set of somatic linker histones.

Chromatin remodeling in somatic cells by Xenopus nucleoplasmin and implications for cell cloning

Somatic cell nuclear transfer (SCNT) involves removing or destroying the nucleus of an unfertilized egg, replacing it with the nucleus of a somatic cell and stimulating this new fused cell to begin dividing and differentiating (see Ref. 39 for review). This technology has allowed for the cloning of animals (such as Dolly the sheep[40]), as well as for the production of embryonic stem cells. However, a limitation of SCNT is the high mortality rate of cloned embryos and therefore, increasing the success rate of SCNT is a major goal in current cloning research.[39] The low efficiency rate is most likely due to an incorrect reprogramming (including that of epigenetic marks such as DNA methylation and histone post-translational modifications) of the donor chromatin which could ultimately lead to aberrant gene expression during development.[41] During gametogenesis, germ cell chromatin naturally undergoes an epigenetic reprogramming process that must be reversed in the donor nucleus during cloning by SCNT. Whether this reversal is caused by many specific or a few general reprogramming factors is not yet known.

Terminally differentiated erythrocytes and sperm cells treated with either *Xenopus* egg extracts or nucleoplasmin alone, undergo nuclear swelling and become transcriptionally active[19] and reactivated for DNA replication.[42,43] Nucleoplasmin is able to remove linker histones from somatic chromatin, yielding a more open and extended chromatin structure.[20] Recently, nucleoplasmin was found to decondense heterochromatin in these cells in an ATP-dependent manner and without the removal of histones.[44] In contrast, ATP is not required for the removal of SNBPs or linker histones during decondensation of sperm or erythrocyte chromatin.[19,20] The undetectable histone displacement and the ATP-dependency could indicate a different mechanism of action. Furthermore, there was an active removal of the centromeric heterochromatin proteins HP1β and TIFβ although their displacement alone was not sufficient to cause chromatin decondensation. Although mouse embryonal F9 cells displayed chromatin decondensation, some other cell lines did not.

In addition, several epigenetic modifications were reported to occur during this process. Histone H3 was phosphorylated at serine 10 and 28 and at threonine 11 and 38 when nuclei were incubated with recombinant nucleoplasmin in the presence of ATP. Additionally, there was an active acetylation of histone H3 at lysine 14 in 50% of the cases. These epigenetic modifications occurred in cell lines regardless of nuclear swelling, suggesting that these modifications were not due to chromatin decondensation. This observation also indicates that the modifications were not the mechanism of action for decondensation. The ability of nucleoplasmin to promote chromatin decondensation and histone modifications, and to displace heterochromatin proteins was dependant on its A2 polyglutamic acid tract in the C-terminal tail. In fact, polyglutamic acid was found to actively promote such processes by itself, although considerably higher amounts of polyglutamic acid were needed when compared with nucleoplasmin, which is in agreement with other studies.[44–46]

The pretreatment of F9 (but not B16 or NIH 3T3) nuclei with nucleoplasmin, or polyglutamic acid, increased the transcription of mouse oocyte-specific genes (H1foo, Msy2, c-Mos and mNpm2) when these nuclei were subsequently injected into *Xenopus* oocytes.[44] Therefore, the use of nucleoplasmin or polyglutamic acid may provide considerable advantages in improving cloning efficiency. In fact, injection of specific concentrations of nucleoplasmin or polyglutamic acid into bovine oocytes during SCNT resulted in an increase in the rate of pregnancy initiation and blastocyst development.[45] However, although microarray analysis of these treated embryos showed an upregulation of over 200 genes relative to untreated embryos, there was no increase in the number of live births suggesting that the nucleoplasmin-mediated reprogramming of gene expression may not be complete.[45]

Overall, there is now plenty of evidence that nucleoplasmin can promote the epigenetic reprogramming of somatic chromatin during SCNT,[45] and this process may act through a different and yet to be determined mechanism than that followed by nucleoplasmin during sperm chromatin decondensation.[44] It will be interesting to see if nucleoplasmin proves to be useful for improving SCNT efficiency in other species and if other nuclear chaperones will have similar results for improving cloning procedures.

Regulation of chromatin condensation during apoptosis: a not so typical function of Xenopus nucleoplasmin

Early in vertebrate development, oocytes are stockpiled and are later depleted over time by apoptotic cell death if they are not fertilized. Gamma-irradiated embryos undergo apoptosis before the mid-blastula transition when the cell cycle is short with no growth phase, but afterwards they are resistant to apoptosis.[47] Morphological changes that take place during apoptosis include condensation of chromatin, shrinking of the nucleus, fragmentation of DNA and, finally, formation of apoptotic bodies. During fertilization in *Xenopus*, nucleoplasmin colocalizes with the sperm chromatin undergoing compositional changes, decondensation and nuclear assembly to form the male pronucleus. Demembranated sperm in *Xenopus* crude egg extracts, upon treatment of with cytochrome c to induce apoptosis, undergo DNA condensation. It was found that nucleoplasmin is excluded from the condensed apoptotic chromatin and is undetectable in the nuclei using this cell-free system.[48] In addition, in *Xenopus* XTC cells induced to undergo apoptosis, nucleoplasmin was excluded from condensed chromatin but remained dispersed within the nucleus.[48]

The specific phosphorylation of tyrosine 124 in the *Xenopus* nucleoplasmin protein was directly linked to apoptotic chromatin condensation, suggesting a new and novel role for nucleoplasmin as a regulator of apoptotic chromatin condensation in *Xenopus*.[48] Nucleoplasmin may act as a general decondensation factor, and dephosphorylation of tyrosine 124 inactivates nucleoplasmin so that apoptotic DNA condensation can occur. This tyrosine residue is conserved in amphibians and even encoded by the same codon, suggesting an important functional constraint on the molecule.[4] The involvement of NPM2 in mammalian apoptosis, in a similar fashion as that presented by amphibians, still needs to be addressed given that tyrosine at position 124 is not conserved in mammals.

NPM1 expression is decreased in human cells undergoing apoptosis and is considered an anti-apoptotic protein.[49] In humans, NPM1 is up-regulated in response to hypoxic stress in normal cells and cancer cells, stopping them from undergoing apoptosis through inhibition of p53 activation, a known binding partner of NPM1.[49] Although both NPM1 and NPM2

have involvements in apoptosis, the processes appear to be quite different.

Nucleoplasmin homologues in mammalian oocytes

Similar to nucleoplasmin in *Xenopus*,[23,50] the mammalian NPM2 ortholog is expressed only in the oocyte.[51] Initially, mammalian NPM2 protein is found in the nucleoplasm of the oocyte. Following fertilization and the breakdown of the germinal vesicle, NPM2 disperses into the cytoplasm until formation of the pronuclei, where it is found until the eight-cell stage. Unlike NPM1 and NPM3, NPM2 is excluded from the nucleolus.

Npm2$^{-/-}$ mice are viable, although the females showed reduced fertility.[51] Oocytes from *Npm2*-null females did not have normal heterochromatin formation surrounding the nucleolus and exhibited decreased nucleolar clearing of acetylated H3 and dispersed nucleolar bodies. Protamine removal and chromatin decondensation proceeded normally following fertilization, leading to the formation of the paternal pronucleus. This is surprising as nucleoplasmin is essential for the correct completion of the early stages just after fertilization in *Xenopus*. The transition from the one- to two-cell stage exhibited an abnormal exit from the first round of meiosis. In vitro, fertilized *Npm2*-null eggs showed a reduced progression to the two-cell stage and in vivo embryos had problems in early development. Therefore, mouse NPM2 is needed for nucleolar heterochromatin organization, chromatin compaction and deacetylation of H3.[51]

It is thus possible that protamine removal is compensated for in *Npm2*-null mice by other NPM family members in the absence of NPM2. However, it is interesting that NPM2 in mammals does not have an A1 acid tract, which in *Xenopus* is important for sperm chromatin decondensation.[25] In addition, it has been shown that human NPM3 may be required for decondensation of sperm chromatin.[17] Oocytes injected with *Npm3* antisense oligonucleotides before fertilization to decrease the level of NPM3 presented a significantly reduced ability to replace protamines with histones in the male pronucleus.[17] However, it may be possible that injection of antisense oligonucleotides may have decreased the expression of other NPM family members that have similar sequences. Therefore, more studies are needed to determine which protein is in fact required for protamine removal in mammals.

In addition to its many other roles, human NPM1 acts as a histone chaperone

Although NPM1 has been implicated in many functions in the cell, it has (like nucleoplasmin) also been shown to bind histones. Like most histone chaperones, NPM1 can bind to H3–H4 tetramers and H2A–H2B dimers, although binding to H3–H4 is preferred.[27,52,53] The H3–H4 tetramers interact with the N-terminal core domain, however, the first C-terminal acid tract (A2) may be needed for H2A–H2B dimer binding.[27,53]

Through the ability to bind histones, NPM1 is able to assemble nucleosomes.[52] Deletion of the 94 C-terminal amino acids resulted in a 40% reduction in this ability, deletion of the C terminus and the second acid tract resulted in a 70% reduction and the N terminus region alone had no ability to form nucleosomes.[53] Thus, in contrast to the histone binding domain, the nucleosome assembly function is localized to the C terminus and last acid tract.

Human NPM1 is able to decondense demembranated *Xenopus* sperm and increases the transcription of acetylated chromatin.[53] Several lysine residues in the N terminus of NPM1 itself are specifically acetylated by p300, making NPM1 the first known histone chaperone amenable to acetylation. There is sufficient acetylated NPM1 in HeLa cells to be detected by Western analysis without treatment with HDAC inhibitors. Acetylated NPM1 is able to increase transcription activity by fivefold over unacetylated NPM1 in an in vitro assay and acetylation of NPM1 increased its binding affinity to acetylated histones. In addition, overexpression of NPM1 increased the transcription of a p53-responsive reporter gene whereas expression of antisense *NPM1* decreased transcription.[53] However, as in the case of amphibian nucleoplasmin, the detailed molecular mechanisms involved in the facilitation of transcription are not completely understood.

Thus, NPM1 likely functions as a histone chaperone in the nucleolus, where highly active transcription creates a need for a histone chaperone to bind any released histones and aid in the assembly of nucleosomes. NPM1 has also been shown to interact with FRGY2a/YB1 and act as a mediator in nucleolar disassembly,[54] emphasizing NPM1s importance for nucleolar structure.

Potential ways in which npm family members may control their multifunctionality

Interaction with other family members

How does NPM1 function in ribosome biogenesis, histone chaperoning and other activities? It could have to do with post-translational modifications, variants or binding to other proteins, possibly even other members of the NPM family. In support of this, NPM1 localized to centrosomes has different immunogenic characteristics (i.e. preferentially recognized by a different antibody) than nucleolar NPM1.[55]

Heterogenous complexes of different NPM family members may allow for regulation of function. This has been implicated for NPM1 and NPM3. Results of a yeast two-hybrid screen indicated that human NPM3 was a major binding partner of human NPM1.[16] This was consistent with the first report of the *Xenopus* homologue of NPM3, NO29, which was immunoprecipitated from cellular extracts with NPM1/NO38.[15] The interacting regions of the human homologues

were narrowed down to amino acids 35 to 90 of NPM1 and 30 to 90 of NPM3, which are located within the core domains. It is yet to be determined whether this association occurs in the form of heterogenous pentamers or if a set of NPM1 and NPM3 homopentamers form decameric complexes.

NPM3 is mainly located in the nucleoli and maintaining this localization requires active rRNA transcription[16] but, unlike NPM1, NPM3 does not seem to associate with rRNA. The overexpression of NPM3 resulted in a decrease in rRNA production and processing and this was dependent on binding to NPM1.[16] Thus, it seems binding of NPM3 to NPM1 may create a complex in which NPM1 can no longer perform its ribosomal biogenesis activities, possibly because it can no longer bind to RNA.[16] Similar to NPM3, NPM1 variants, which lack the nucleic-acid-binding domain, inhibit ribosome biogenesis.[56,57] Thus, variants of NPM1 as well as other NPM family members can act as binding partners to modulate its cellular activities. So far no studies have been done to assess the effect of the NPM1–NPM3 association on histone chaperone function.

Post-translational modifications of NPM proteins: phosphorylation, acetylation, glutamylation, polyribosylation

Post-translational modifications, especially phosphorylation, are common mechanisms for controlling the function of proteins. NPM1 is phosphorylated by several kinases at multiple sites. One example is phosphorylation of Threonine199 by CDK2/cyclin E, which is involved in the regulation of centrosome duplication[58,59] and targets NPM1 to nuclear speckles, enhances RNA-binding and represses pre-mRNA splicing.[60] Thus, phosphorylation seems to regulate NPM1 activity, a process that takes place in a cell-cycle-dependent manner. Among other sites, Thr199 as well as Thr219, Thr234 and Thr237 were identified as potential phosphorylation targets for cyclin B/cdc2 during mitosis.[57] In addition, NPM1 is acetylated by histone acetyltransferase p300 resulting in an increase in transcription of a p53-responsive synthetic reporter gene.[53]

NPM1 was found to co-precipitate with components of a neuronal gene repressor complex.[61] The components of this repressor complex, including NPM1, are modified by polyribosylation, which allows for their dissociation from the complex and gene promoter. Poly(ADP)ribosylation may not be a general modulator of NPM1 activity, but may instead be specific to this example, as all of the other proteins in the repressor complex are also modified.

Phosphorylation is required for the biological activity of nucleoplasmin, with the protein having a higher degree of phosphorylation in eggs than oocytes.[62] The exchange of H2A–H2B heterodimers for SPs by nucleoplasmin mediated by the phosporylation of up to 14–20 phosphates per nucleoplasmin monomer.[22,63] Phosphorylation of nucleoplasmin during maturation into the egg releases the highly negatively charged unstructured C termini of the nucleoplasmin pentamer, which most likely provides the complex with a more open structure amenable for sperm chromatin remodeling (Fig. 4). It is important to note that, while the histone interactions involve stereospecific constraints (both in their interaction with nucleoplasmin and with the negatively supercoiled DNA of the nucleosome), binding of protamines is mediated by electrostatic interactions. Indeed, the nucleoplasmin region responsible for the binding of protamines seems to be able to bind generically to polycationic chromosomal proteins (protamines, SPs, histone H1), which are part of the 'accessible' linker DNA connecting nucleosomal structures. As mentioned above, phosphorylation of nucleoplasmin at tyrosine 124 further acts as a switch in chromatin condensation during apoptosis.

NAP1 and nucleoplasmin are both characterized by exhibiting acid tracts in their C-terminal domains. Within this region, NAP1 has two glutamylation motifs, which may allow for a reversible increase in the charge of its C-terminal tail by the addition of poly-glutamate side chains.[64] Nucleoplasmin has also been observed to be amenable to glutamylation.[65] The addition of acid side chains could alter the binding specificity of these chaperones to different proteins.

Conclusion

From an evolutionary perspective, the members of the NPM family follow a functional diversification process and are subject to purifying selection. NPM1 and NPM3 are more closely related and appear to have differentiated later during evolution than NPM2,[4] which represents the most specialized NPM lineage and acts as a chaperone in oocytes and eggs. The understanding of the role played by NPM2 in H2A and H2B storage,[66] as well as in sperm chromatin remodeling through the removal of SNBPs,[8,62] has more recently being expanded to hypothetically include assisting in nucleosome assembly during early development until the MBT[37,67] and acting as a general decondensation factor, even inhibiting chromatin condensation during apoptosis in *Xenopus*.[48] On top of numerous functions fulfilled by NPM1 in the cell lies its role as a histone chaperone in the nucleolus. The different roles played by this protein are regulated by binding partners, including NPM3. Post-translational modifications are also critical in the regulation of NPM family members and allow them to perform different activities in the cell. A better understanding of the locations and temporal occurrence of PTMs is still needed, especially in the case of nucleoplasmin, which is known to become heavily phophorylated,[62] but the amino acids modified have not been experimentally determined.

Very little is known about the function played by nuclear chaperones in remodeling mammalian sperm chromatin following fertilization. It is not clear which chaperone is responsible for the removal of protamines in this taxonomic

group, indicating that more research is needed in this field. Given that NPM2 is one of only a few proteins that are known to be required for the transition from the one- to two-cell stage in mammalian development,[51] further research into NPM2 could be important for treatment and diagnosis of infertility in humans. Similarly, characterization of the activity of NPM3 following fertilization, which may be responsible for sperm chromatin decondensation in mammals,[17] is imperative and should be a focus of future research in this field.

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