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Insights into the regulation of neuronal viability by nucleophosmin/B23

Jason A Pfister[1,2] and **Santosh R D'Mello**[2]

[1]Department of Biological Sciences, University of Texas at Dallas, Richardson, TX 75080, USA

[2]Department of Biological Sciences, Southern Methodist University, Dallas, TX 75275, USA

Abstract

The vastness of the neuronal network that constitutes the human brain proves challenging when trying to understand its complexity. Furthermore, due to the senescent state they enter into upon maturation, neurons lack the ability to regenerate in the face of insult, injury or death. Consequently, their excessive death can be detrimental to the proper functioning of the brain. Therefore, elucidating the mechanisms regulating neuronal survival is, while challenging, of great importance as the incidence of neurological disease is becoming more prevalent in today's society. Nucleophosmin/B23 (NPM) is an abundant and ubiquitously expressed protein that regulates vital cellular processes such as ribosome biogenesis, cell proliferation and genomic stability. As a result, it is necessary for proper embryonic development, but has also been implicated in many cancers. While highly studied in the context of proliferative cells, there is a lack of understanding NPM's role in post-mitotic neurons. By exploring its role in healthy neurons as well as its function in the regulation of cell death and neurodegeneration, there can be a better understanding of how these diseases initiate and progress. Owing to what is thus far known about its function in the cell, NPM could be an attractive therapeutic target in the treatment of neurodegenerative diseases.

Keywords

Nucleophosmin; neurons; neurodegeneration; nucleolus; Huntington's disease; cell cycle

Introduction

Nucleophosmin 1 (NPM1), also known as nucleophosmin (NPM), B23, numatrin or NO38, is a nucleolar phosphoprotein involved in an array of physiological processes including ribosome biogenesis, cell cycle regulation, centrosome duplication, genomic stability, apoptosis and can function as a molecular chaperone. It has been implicated in leukemias and lymphomas and is one of the most frequently altered genes in haematopoietic tumors. Further, it is over-expressed in many tumors and as such has been suggested as a marker for a range of carcinomas.[1,2] The entire region to which the *NPM1* gene maps is also seen

Corresponding author: Santosh R. D'Mello. sdmello@smu.edu.

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deleted in many cases of *de novo* myelodysplastic syndrome, non-small cell lung carcinoma and acute myeloid leukemia.[1,2]

To date, the majority of work surrounding NPM has investigated its role in proliferative cells (summarized in Table 1). Interestingly, it is highly expressed in the brain but little is understood about its function in post-mitotic neurons. Here, we examine what is known about NPM function and how it may relate to the regulation of neuronal viability. While NPM has been implicated in many cellular functions, this review will focus on its role in the nucleolus, cell proliferation, apoptosis and development. We will conclude by discussing what is currently understood about its function in neurons and its potential in neurodegenerative diseases.

The nucleophosmin/nucleoplasmin family

NPM was the first member identified of the nucleophosmin/nucleoplasmin family of proteins, which also includes NPM2 and NPM3. The human *NPM1* gene is composed of 12 exons at chromosome 5q35.1 and is transcribed into three isoforms: isoform 1 corresponds to the longest and full length NPM protein (294 amino acids), isoform 2 lacks an in-frame exon (exon 8) and encodes a smaller protein of 265 amino acids and isoform 3 (also known as B23.2) uses an alternative 3′-terminal exon (exon 10) that encodes a 259 amino acid protein with a distinct C-terminus. The NPM protein is characterized by four domains: an N-terminal oligomerization domain (residues 1–120) that contains two nuclear export signals (residues 42–47 and 94–102) and is responsible for its multimeric state and interaction with other proteins, a central region containing two acidic stretches (residues 121–132 and 160–188) and a bipartite nuclear localization signal (residues 141–157), a basic domain important for binding nucleic acids (residues 189–243) and a C-terminal aromatic domain (residues 244–294) that contains a nucleolar localization signal (resides 288–290).[3,4] Ubiquitously and highly expressed, NPM is predominately nucleolar but does shuttle to the nucleoplasm and cytoplasm. NPM2, also known nucleoplasmin 2, is a nuclear protein that is primarily found in oocytes but will not be discussed here. Similar to NPM, NPM3, also known as nucleoplasmin 3, is ubiquitously expressed and primarily localizes to the nucleolus. However, NPM3 is a smaller protein of 178 amino acids that is characterized by an N-terminal oligomerization domain, a C-terminal aromatic domain responsible for its nucleolar localization and, unlike NPM, does not contain a central acidic or basic domain.[5]

Nucleolus and ribosome biogenesis

The nucleolus is a non-membrane bound region inside the nucleus responsible for ribosome biogenesis and is divided into three compartments that have designated roles: the fibrillar center (FC, pre-rRNA synthesis), the dense fibrillar component (DFC, pre-RNA processing) and the granular component (GC, ribosome assembly). Ribosomal DNA (rDNA) consists of tandem repeats on the short arms of chromosomes 13, 14, 15, 21 and 22 that make up a region called the nucleolus organizer region (NOR) around which the nucleolus forms. Each repeat contains the 47S pre-rRNA that is transcribed by RNA polymerase I and consists of a promoter and 5′- and 3′-external transcribed spacers (ETS) that flank 18S, 5.8S and 28S

exons which are each separated by an internal transcribed spacer (ITS), referred to as ITS1 and ITS2. The 5S rRNA is transcribed in the nucleoplasm by RNA polymerase III.

Once transcribed, the pre-rRNA is processed through cleavage of the ITS regions rather than a splicing event. Cleavage of ITS1, which separates the small 40S subunit 18S rRNA from the large 60S subunit 5.8S and 28S rRNAs has been proposed to be mediated by a complex requiring both endo- and exonuclease activity.[6] NPM localizes in the GC and at the border of the DFC and has been reported by Savkur et al. to contain endoribonuclease activity specific for ssRNA and preferentially cleaves ITS2 of the 32S rRNA intermediate to create a mature 28S rRNA.[7] Indeed NPM interacts with both 47S and 28S rRNAs but not 18S.[8] NPM may not only be important for maturation, but also quality control of rRNA. PARP-1 and PARP-2, proteins involved in the DNA-damage response, localize in the nucleolus independent of one another and interact with NPM, but are not required for active rRNA transcription.[9] While this localization is only modestly reduced following oxidative stress, they are, however, extruded to the nucleoplasm following RNA polymerase I inhibition. PARP-1 and PARP-2's need for transcription to remain nucleolar suggest that they may act as surveyors for strand breaks.[9] NPM has additionally been found to interact in the nucleolus with the DNA repair enzyme, APE1/Ref1, and here too requires rRNA transcription.[8] APE1 is a protein critical to the cell that is involved in base excision repair and acts as an apurinic/apyrimidinic (AP) endonuclease. NPM can stimulate APE1 endonuclease activity on abasic dsDNA but reduces activity on ssRNA.[8] With APE1 knockdown, while rRNA transcription is not affected, translation and recovery from oxidative stress are impaired. These two studies suggest that NPM is not only involved in the production of ribosomes, but it further helps coordinate their quality.

As chromatin is duplicated and the cell prepares for division during mitosis, transcription and translation are halted. During mitosis, the nucleolus is disassembled and transcription of rRNA and processing of pre-rRNA is blocked through regulation by the Cyclin B/CDK1 complex and protein phosphatase 1 (PP1).[10] CDK1 phosphorylates NPM at the beginning of mitosis at Thr199 and Thr234/237, resulting in inhibition of NPM RNA binding activity.[11] During anaphase, the activity of CDK1 decreases, which is also a time when NPM is dephosphorylated. PP1, which is itself regulated by CDK1, has been implicated in this dephosphorylation event.[12] During mitosis, inhibition of NPM's RNA binding activity is also observed through heterodimerization with B23.2 in the nucleoplasm.[11] As mentioned, B23.2 is an NPM isoform that utilizes an alternate 3′-terminal exon, thereby lacking the C-terminus of NPM important for DNA/RNA binding. Interestingly, similar to B23.2, NPM3 lacks this same C-terminal domain and can localize in the nucleoplasm, interact with NPM and inhibit ribosome biogenesis through interference of an NPM/28S interaction.[13] The inhibition of NPM's rRNA processing can further be regulated by casein kinase 2 (CK2). CK2 phosphorylates NPM at Ser125, which is located in one of NPM's acidic stretches, a region that, along with its N-terminal oligomerization domain, is essential for its chaperone activity.[14] Thus, CK2 phosphorylation promotes the dissociation of substrates bound to NPM.[15] Furthermore, this phosphorylation during interphase enhances NPM movement through the nucleolus.[12] NPM is necessary not only for pre-rRNA processing, but for the transport and nuclear export of both 40S and 60S ribosomal subunits.[3,16,17] CK2, along with CDK1, phosphorylation may help reduce NPM's nucleolar localization during mitosis and

interfere with its binding to rRNA or ribosomal components, thereby inhibiting ribosome biogenesis.

The tumor suppressor protein ARF, otherwise known as $p14^{ARF}$ in humans and $p19^{ARF}$ in mice, is an *a*lternate *r*eading *f*rame of the locus encoding the broad-spectrum CDK inhibitor, CDKN2A/$p16^{INK4A}$. It is an upstream component that positively regulates p53 activity and is known to inhibit cell proliferation in both a p53-dependent and -independent manner.[18] ARF primarily localizes to the nucleolus and has been implicated in the regulation of ribosome biogenesis. Indeed, $p14^{ARF}$, along with topoisomerase I, co-precipitates with the human rRNA gene promoter.[19] Apicelli et al. have suggested that ARF may act to monitor basal ribosome production as knockdown leads to increases in both ribosome biogenesis and protein production.[20] In line with this, ARF inhibits the nucleolar import of TTF-1, the RNA polymerase I transcription termination factor, which is a job entrusted to NPM.[21] Nucleoplasmic accumulation of TFF-1 will result in its ubiquitination by MDM2.[22] Interestingly ARF, which is known to target and inhibit MDM2, competes with MDM2 for TTF-1 interaction, and thus here too may act as a monitor as too much TTF-1 can inhibit ribosome biogenesis.[22] ARF may further regulate ribosome production through sumoylation of NPM, a modification that results in NPM's nucleolar localization and protection against capsase-3-mediated cleavage,[23] but also prevents 28S mRNA maturation.[24] This sumoylation event can be reversed by Senp3, a SUMO2/3 protease. Indeed, knockdown of Senp3 inhibits the conversion of 32S rRNA to 28S, a process further observed with NPM depletion.[24] ARF and Senp3 both interact with NPM and antagonize one another to regulate its sumoylation, with ARF triggering phosphorylation and then ubiquitination of Senp3.[25]

Centrosome duplication

Upon exit from the G_1 phase of the cell cycle, centrosomes, which serve as the main microtubule-organizing center, must replicate. Failure to do so leads to an inability to properly form the mitotic spindle during prophase and thus a dysfunctional cell division. The G_1/S transition is regulated by Cyclin E/CDK2 phosphorylation. Okuda et al. have shown that NPM is bound to unduplicated centrosomes and is a target of Cyclin E/CDK2.[26] Phosphorylation at Thr199 results in its dissociation thereby leading to centrosome duplication. NPM is found reassociated with centrosomes during mitosis. Interference in this phosphorylation, such as with a phosphorylation-deficient Thr199Ala mutant, results in NPM remaining with the centrosome and a blockage in duplication.[26,27] Thr199 phosphorylation by CDK2 further regulates an interaction between NPM and Rho-associated protein kinase II (ROCK II). ROCK's are serine/threonine kinases that act on the cytoskeleton to regulate the morphology and movement of the cell. However, ROCK II has been found to localize to centrosomes and physically interacts with NPM.[28] Upon phosphorylation by CDK2, NPM displays a higher affinity for ROCK II and their interaction greatly increases ROCK II kinase activity. Moreover, knockdown of ROCK II showed a suppressive effect on centrosome duplication while expression of a constitutively active form promoted it.[28] This pathway further requires RhoA and RhoC priming of ROCK II; however, a downstream effector remains elusive.[29]

Subsequent to discovering CDK2 targeting of Thr199, three independent studies concurrently linked NPM to the Crm1 network,[3,4,30] two of which examined a connection to centrosomal duplication.[4,30] The Ran-Crm1 complex regulates nuclear export of proteins that contain leucine-rich nuclear export signals in a GTP-dependent manner.[31] Crm1 is reported to play a role in the regulation of centrosome duplication and mitotic spindle assembly, where its inhibition leads to centrosome amplification and multipolar spindles.[32] NPM is associated with, and localized between, pairs of centrioles of unduplicated centrosomes, suggesting a role in centriole pairing, and here co-localizes with Crm1.[4,30] Inhibition of Crm1 through leptomycin B treatment results in increased Cyclin E at the centrosome, NPM dissociation and centrosome duplication.[4,30] As found by others,[11] NPM associates with mitotic centrosomes which is thought to be via interaction with the Ran-Crm1 complex, thereby preventing reduplication, and remains there until phosphorylated by CDK2.[4,26,30] Further evidence to support these findings indicates that NPM is a target of polo-like kinase 1 (Plk1) and polo-like kinase 2 (Plk2) phosphorylation at Ser4 during mitosis and centriole duplication in S-phase, respectively.[33,34] Blockage of Plk1 phosphorylation results in abnormal centrosome numbers, fragmented nuclei and incomplete cytokinesis,[33] whereas Plk2 phosphorylation causes interference with centriole reduplication and diluted centriole numbers.[34]

Oxidative stress and DNA damage

The regulation of p53 mediates the G_1/S transition and apoptosis in the face of oxidative stress and DNA damage. Under normal conditions, p53 levels are rapidly turned over by degradation through interaction with the E3 ubiquitin ligase, MDM2. Upon cellular insult, such as oxidative stress resulting in DNA damage, MDM2 is inhibited either by auto-ubiquitination or interaction with other proteins such as ARF. A now free p53 can initiate transcription of DNA repair machinery as well as proteins that will halt cell cycle progression. An inability for the cell to correct itself will result in p53-mediated apoptosis.

Through interaction with each member, and depending on the state of the cell, NPM can be a positive or negative mediator of the ARF/MDM2/p53/p21 pathway. In both normal and malignant hematopoietic cells, NPM overexpression inhibits p53 to protect the cell against stress-induced apoptosis.[35] Further, under an oncogenic state, MDM2 can interact with ARF in the nucleolus, allowing for a nucleocytoplasmic shuttling of an ARF-sequestered NPM and progression of the cell to S-phase.[36] However, in response to UV-induced damage, NPM translocates from the nucleolus to the nucleoplasm and interacts with and inhibits MDM2, thereby freeing p53.[37] In line with this, NPM can interact with and stabilize p53, leading to an increase in p53 transcriptional activity[38] as well as regulate its response to UV stress through competition for phosphorylation by UV-activated ATR.[39] One of p53's most well-known transcriptional targets is p21(WAF1/CIP1). Xiao et al. have shown that, similar to p53, NPM and p21 directly interact. p21 is a short-lived protein and NPM contributes to its stability by negatively regulating its ubiquitination and thus degradation by the proteasome, thereby providing an anti-proliferative effect.[40] Following DNA damage, both p53 and p21 target GADD45α, a protein that can translocate into the nucleus and arrest the cell cycle at the G_2/M transition by interfering with the Cyclin B1/CDK1 complex.[41,42] NPM can regulate this nuclear import, thus influencing a G_2/M arrest.[43] Interference with

this interaction or knockdown of endogenous NPM blocks GADD45α import, leading to an inability to arrest the cell cycle.

Perhaps NPM's closest interaction in p53 regulation is ARF, and interestingly this partnership is both cooperative and antagonistic. While found in the nucleoplasm, ARF predominately localizes to the nucleolus. Due to its abundant nature, only a small portion of NPM binds ARF while most cellular ARF is bound to NPM.[44] In the nucleolus, ARF is stabilized and inhibited from degradation through its interaction with NPM. Indeed, ablation of NPM results in decreased ARF stability.[45,46] In cases of acute myeloid leukemia that express an NPM with a mutated C-terminus that results in its cytoplasmic relocalization, ARF was also found to be relocalized to the cytoplasm and less stable.[47] As discussed earlier, ARF regulates NPM sumoylation, a modification important for its centrosomal and nucleolar localization as well as its stability.[23] However, interestingly, it can also induce NPM degradation by promoting polyubiquitination.[48] NPM's interaction with ARF, which is dependent on NPM oligomerization, occurs in the domains of ARF responsible for its nucleolar localization and MDM2 binding.[44,49] Thus, while nucleolar retention stabilizes ARF, it also inhibits its ability to target MDM2. The NPM/ARF interaction can be disrupted by DNA damage as well as phosphorylation of NPM at Ser48 by Akt, both of which lead to ARF's nucleoplasmic transition and interaction with MDM2.[49,50] Following DNA damage, cJun can interact with NPM and cause NPM and ARF redistribution, an event that requires JunB, JNK activation and its phosphorylation of cJun at Thr91 and Thr93.[51]

The E2F1 transcription factor is a critical regulator of cell cycle progression past the DNA damage checkpoint in G_1. Under non-optimal growth conditions, a hypophosphorylated pRb will bind to and inhibit E2F1, thereby preventing E2F1-mediated transcription. This hold is relinquished through phosphorylation of pRB by Cyclin D/CDK4-6 in G_1, followed by Cyclin E/CDK2 in the transition to S-phase. Hyperphosphorylation causes a conformational change in pRb, separating its pockets A and B and disrupting the E2F1 binding site. pRb will remain in this inactive state until it is dephosphorylated by phosphatases such as PP1 and PP2A after mitosis and in response to oxidative stress, respectively.[52,53] Takemura and colleagues have shown that upon hyperphosphorylation, pRb localizes to nucleoli during late S-phase/early G_2 and here was associated with NPM.[54] This interaction occurred through pRb's pocket A region and was promoted by, and dependent on, pRb phosphorylation. An unphosphorylated pRb or interference with pocket A inhibits pRb's translocation.[54] Interaction with pRb also requires sumoylation of NPM by ARF Lys263.[23] E2F1 transcriptional activity is greatly increased in the presence of wild-type NPM; however, it is abolished with an NPM Lys263Arg mutant.[23] Furthermore, NPM has been implicated in DNA repair in an E2F1-dependent manner. Following UV-induced damage, NPM is phosphorylated at Thr199 and Thr 234/237, leading to an increase of E2F1 mRNA.[55] NPM then associates with pRB and interfers with its repression of E2F1 transcription as seen by an increase of E2F1 at the promoters as well as increased expression of known E2F1 targets in DNA repair, XPC and DDB2.[55]

Regulation of apoptosis

Signaling pathways

NPM has been connected to the pro-survival nature of the PI3-K/Akt and MAPK/ERK signaling pathways. Overexpression of NPM in PC12 cells is protective against apoptosis through physical interaction with nuclear $PI(3,4,5)P_3$ and can mediate the anti-apoptotic effects of NGF. This occurs through inhibition of *C*aspase-*A*ctivated *D*Nase (CAD),[56] a protein responsible for the introduction of strand breaks in and subsequent fragmentation of DNA, a hallmark of apoptosis. NPM interacts with CAD that has been freed from its inhibitor, ICAD, thereby preventing CAD-mediated DNA fragmentation. This is dependent upon $PI(3,4,5)P_3$ as an NPM mutant that fails to bind $PI(3,4,5)P_3$ fails to bind CAD.[56] Nucleolar dislocation of NPM is subject to caspase-3-mediated cleavage, leading to proteasomal degradation that can be prevented by the binding of ATP to Lys263.[57] ATP depletion or a Lys263Asp mutation, which is also defective in $PI(3,4,5)P_3$ binding, will localize NPM to the nucleoplasm and render it unstable. As such, ATP binding and $PI(3,4,5)P_3$, are necessary for NPM's anti-apoptotic effects following expression in PC12 cells.[57,58] Interestingly, Lys263 is also sumoylated by ARF, blockage of which interferes with $PI(3,4,5)P_3$ interaction and results in DNA fragmentation as well as nucleoplasmic localization and degradation of NPM.[23]

$PI(3,4,5)P_3$ preferentially binds NPM in the nucleus and can regulate, by interfering with, an interaction between NPM and Akt.[59] Nevertheless, NPM interacts with phosphorylated/active and nuclear translocated Akt upon growth factor stimulation.[60] This interaction occurs between NPM's C-terminus (residues 239–294) and Akt's PH domain, which thereby further protects NPM from caspase-3 mediated degradation as well as cellular DNA fragmentation. Indeed, Akt knockdown in hippocampal cultures leads to NPM cleavage.[60] The cell expresses three Akt isoforms (Akt1-3) and NPM interacts specifically with Akt2. As stated, sumoylated NPM is protected from degradation. While an unsumoylated NPM Lys263Asp interacts more strongly with, and is protected by, Akt2, this could be due to regulation of NPM nucleolar localization.[60] Consistent with the survival-promoting effect in PC12 cells, NPM and Akt2 work together to promote tumorigenesis of a human lung adenocarcinoma cell line.[61] Thus, while the effects of an NPM/Akt2 interaction may overlap across cell types, differences may occur depending on the cell's proliferative nature.

NPM has additionally been shown to be regulated by the MAPK pathway. NPM is downregulated during differentiation as well as apoptosis of cell lines, cellular processes that can be blocked by its overexpression.[62–64] Upon induction of megakaryocytic differentiation of K562 cells by TPA treatment, NPM downregulation, which can be blocked by proteasome inhibitors, is due to the activation of MAPK/ERK signaling specifically by nPKC.[65] Under retinoic-acid (RA) induced differentiation NPM is recruited to RA-induced promoters, including NPM's own, by AP2α and acts as a co-repressor in conjunction with HDAC1/2, thereby resulting in a chromatin structural change.[66] Interestingly, Inder et al. have reported that NPM can regulate the MAPK pathway through interaction with K-Ras at the plasma membrane.[67] K-Ras is a membrane bound GTPase that can initiate signaling of both the PI3K and MAPK pathways. NPM interacts with both GDP and GTP bound K-Ras;

however, this interaction is increased upon EGF stimulation and leads to K-Ras stabilization and increased clustering, a process that increases the activation of MAPK signaling.[67] One caveat to these studies is comparing the examination of NPM function in MAPK signaling in proliferative and differentiating cells. Stimulation of this pathway as shown by Inder and colleagues would indicate a protective function for NPM; however, it is not stated if increased MAPK signaling in turn resulted in decreased NPM expression. As K-Ras has multiple targets, it is possible that the interaction leads to PKC-independent MAPK signaling.

Cellular localization

NPM is predominately a nucleolar and nucleoplasmic localized protein, where it provides a pro-survival effect for healthy proliferative cells. Nucleocytoplasmic shuttling has also been described, and while important for the transport of ribosomal subunits for ribosome assembly, cytoplasmic localization of NPM has indicated a potential role in the regulation of cell viability. Acute myleogenous leukemias express a mutant of NPM, termed NPMc+, that localizes to the cytoplasm.[68] NPMc+ contains a mutation in exon 12 leading to a Trp288Cys substitution, which is a residue important for NPM's nucleolar localization as well as nucleocytoplasmic shuttling. NPMc+ results in the cell being more prone to apoptosis following bortezomib and arsenic trioxide treatments, compounds that induce reactive oxygen species. Mutation of this residue to alanine allows for relocalization of NPMc+ to nucleoli and a reduction in the cell's sensitivity to these drugs.[69]

Translocation to the cytoplasm can further be induced by nucleolar localized NF-κB, a protein complex known to regulate apoptosis among many other processes. This complex is composed of hetero- or homodimers consisting of combinations of five proteins: p50, p52, RelA (p65), RelB and c-Rel. NF-κB is normally sequestered and inhibited in the cytoplasm by IκBα/β; however, upon dissociation from IκBα/β, it can translocate into the nucleoplasm to regulate transcription. A few studies have described a nucleolar localization pattern for NF-κB. Stark et al. have shown that upon exposure to an NF-κB pro-apoptotic stimulus, aspirin, as well as serum withdrawal and UV–C radiation, RelA is sequestered in nucleoli, but is excluded in response to TNF and TRAIL.[70] Inactivation of Cyclin D1/CDK4 either pharmacologically or in a p38-mediated manner can also induce nucleolar targeting of RelA and apoptosis.[71,72] Nucleolar NF-κB results in decreased basal levels of NF-κB transcriptional activity as well as apoptosis, which is reversed by retention in the nucleoplasm or extrusion from nucleoli.[70] This localization of RelA results in a translocation of NPM from the nucleoli to the cytoplasm. The induction of apoptosis is Bax-mediated and dependent on NPM chaperone activity.[73] While NF-κB is necessary for p53-mediated apoptosis,[74] a molecule that can induce the expression of Bax, p53 is not required for aspirin-induced death.[75] However, in neural precursor cells treated with staurosporine, apoptosis is p53- and Bax-dependent.[76] p53 translocates to the cytoplasm in a Crm1-independent manner and co-localizes with activated Bax on mitochondria.[76] Staurosporine treatment further promotes an interaction of NPM with p53 and active Bax in the cytoplasm, suggesting the formation of a complex that can induce apoptosis in these cells.[76] Interestingly, Dhar and St. Clair reported that in TPA-treated skin epithelial cells, while overexpression of NPM can increase expression of p21 and Bax, proteins thought to be

involved in the mechanism by which TPA induces cell death, it can block apoptosis by interfering the with mitochondrial localization of p53.[77,78] While inconsistencies among these findings are the roles played by p53 and NPM, the differences seen can be due to the cell type and apoptosis-inducing stimulus used. Nevertheless, there is a clear indication that a cytoplasmic-localized NPM has a role in the regulation of apoptosis. This is in agreement with results from our lab showing that expression of a cytoplasmic NPM, due to deletion of its bipartite nuclear localization signal, is toxic to otherwise healthy cerebellar granule neurons (CGN). Forced retention in the nucleus due to a mutation of its nuclear export signal reverses this toxicity.

The role of NPM in development

Much knowledge of the role of NPM during development has come from the analyses of knockout and hypomorphic mice. Grisendi et al. generated a hypomorphic NPM mutant series ($NPM^{+/-} < NPM^{hy/hy} < NPM^{-/-}$) of mice.[79] While $NPM^{+/-}$ mice are viable and thrive, both $NPM^{-/-}$ and $NPM^{hy/hy}$ mice show dysfunction in embryonic development and die *in utero* at ~E12 and ~E16.5, respectively. By day E9.5, $NPM^{-/-}$ mice display deficiencies in development of the telencephalic region of the brain while the mesencephalic and anterior metenchepalic areas are largely maintained.[79] These mice additionally show markedly increased apoptosis, especially in the neural tube, and die due to aberrant organogenesis resulting from severe anemia from primitive haematopoiesis.[79] $NPM^{hy/hy}$ mice, which have a reduced function in NPM to ~10–30% of wild type, display a similar, yet less dramatic, phenotype. Consistent with earlier *in vitro* work mentioned earlier, a lack of NPM shows unrestricted centrosome duplication and genomic instability.[79] Concurrently, Colombo et al. created a different $NPM^{-/-}$ mouse that was also mid-stage embryonic lethal (~E10).[45] These mice show an increase in p53-mediated apoptosis as part of the DNA damage response as opposed to ARF activation. NPM was found, instead, to be downstream of ARF and critical for ARF's nucleolar localization and stability.[45] $MEF^{-/-}$ cells cultured from the mice were capable of growth in cell culture, provided p53 expression was additionally knocked out, and both the $MEF^{-/-}$ cells and $NPM^{-/-}$ embryos show an increase in cell proliferation compared to wild type littermates.[45] Interestingly, while increase in growth speed is additionally observed in $NPM^{-/-}$ $p53^{-/-}$ double mutant MEFs, Grisendi and colleagues found that embryonic lethality was not rescued.[79] These findings are in line with those of another study that found downregulation of NPM in hypothermia-induced neural tube defects. siRNA-mediated knockdown of NPM in neural stem cells results in a decreased cell proliferation rate, an increase in apoptosis as well as increased p53 and p21 expression.[80]

The role of NPM in neurons

The information described until now has mostly concerned NPM's role in proliferative cells (summarized in Table 1). However, even though it is highly expressed in the brain, its function in post-mitotic neurons remains obscure. To date, only a handful of reports have detailed a role for neuronal NPM (summarized in Table 2). Here, we highlight these recent findings and discuss potential future directions to hopefully shed light on NPM's involvement in the regulation of neuronal viability.

As previously described, overexpression of NPM provides a protective effect against apoptosis through nuclear PI(3,4,5)P_3 in nerve growth factor-treated PC12 cells, a treatment that can differentiate this cell line into neuronal-like cells. In agreement with this effect, NPM appears to positively regulate neuronal viability after spinal cord injury and in the face of excitotoxicity.

The hematopoietic growth factor, granulocyte colony-stimulating factor (G-CSF), has been found in the adult brain and spinal cord.[81] Guo et al. have shown that treatment of mice with a hemisection of the right hemicord with G-CSF improves the survival of neurons in the spinal cord and NPM is found to be upregulated.[82] Inhibition of NPM pharmacologically with NSC348884 partially blocked this protection *in vitro* and resulted in p53 upregulation, an effect shown by Qi et al. who first characterized the compound.[82,83]

The overexcitement of neurons through excessive stimulation of glutamate receptors leads to high levels of Ca^{2+} influx in what is referred to as excitotoxicity, which ultimately results in the death of the cell. Apoptosis in this manner has been implicated in neurological conditions ranging from spinal cord and traumatic brain injuries to neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). Excitotoxicity both *in vitro* and *in vivo* can be induced by treatment of glutamate receptor agonists such as glutamate, NMDA or kainic acid (KA). In primary cortical neurons cultured from presenilin 1 (PS1) M146V mutant knock-in mice, a protein whose mutations underlie familial AD, NPM mRNA expression is decreased following glutamate treatment.[84] This was not seen in glutamate-treated cultures from $FE65^{-/-}$ or $APOE^{-/-}$ mice.[3] Further, NPM expression can protect against NMDA-mediated excitotoxicity by inhibiting the SIAH1-GAPDH death cascade in primary neuronal cultures as well as *in vivo*.[85] Protection occurs through binding both SIAH1 and GAPDH, thereby interfering with their interaction and blocking SIAH1 E3-ligase activity.[85] This is dependent on S-nitrosylation of NPM at Cys275 by GAPDH as a Cys275Ser mutation or shRNA against NPM blocked this protective effect.[85] In line with both of these studies, NPM protein but not mRNA is downregulated in degenerating neurons of the hippocampal CA1 region following KA-induced excitotoxicity.[86] Overexpression in the SH-SY5Y cell line protected against KA-induced injury. In these same cells, NPM downregulation by siRNA results in upregulation of p53. However, KA treatment into $p53^{-/-}$ mice, in which there is minimal death, showed no change in NPM levels compared to the control. As such, NPM downregulation-induced death following KA treatment appears to be p53-independent, possibly involving Bax.[86]

Aberrant cell cycle induction in neurons

The small body of working describing the function of NPM in neurons and the neuronal-like PC12 cells has suggested a pro-survival role. However, its necessity for centrosome duplication and interactions with other proteins that are important for cell cycle progression suggest a more complicated role for neuronal NPM. Proper control of the cell cycle is critical for proliferative cells and misregulation can lead to its inhibited progression, resulting ultimately in apoptotic death. While vital for development of the nervous system, neurons enter a senescent stage upon maturation and become post-mitotic. As described

above, NPM is critical for the proper development of the telencephalon, the embryonic structure that becomes the cerebrum of the brain. While others have described a protective role, we find that overexpression is toxic to healthy primary CGNs kept alive by high levels of potassium, a model that mimics the pro-survival nature of developing neurons. Interestingly, this toxicity is blocked by CDK inhibition.

A growing theory to explain the excessive apoptosis that leads to neurodegenerative diseases is through a neuron's aberrant re-entry into the cell cycle.[87] Indeed, an increase in the expression of cell cycle regulatory proteins and cell cycle activation are seen in many of these diseases, including AD, PD and amyotrophic lateral sclerosis (ALS), as well as a chemically induced animal model of HD.[88–91] What is unclear, however, is if the increases in these proteins are enough to trigger excessive neuronal death and degeneration or are by-products of some other insult. One such insult, for instance, that could drive this phenomenon is oxidative stress, a form of damage to the cell that can induce cell cycle and is well-known to be implicated in aging and neurodegenerative diseases.[88–90] As a result, antioxidants have become a popular therapeutic approach.[92] Endogenously, the superoxide dismutases (SODs) are vital antioxidants required for cell health. Loss of one member, the mitochondrial MnSOD, or SOD2, is found in many neurological pathologies as well as age-related cognitive decline.[93] Dhar et al. have shown that NPM acts as a co-activator with NF-κB, specifically p50 and p65 (RelA), to induce the expression of MnSOD in the presence of PMA (PKC-dependent) and cytokines (TNFα and IL-8).[94] This effect is NPM-dependent as antisense NPM reduces MnSOD gene transcription and endogenous protein levels.[94]

One protein well-known to respond to DNA damage and regulates cell cycle progression is E2F1. Upon being freed from pRb inhibition, E2F1 can regulate the expression of many apoptotic regulatory proteins including, but not limited to, cdc2, ARF, p53, PUMA, BIM, SMAC/DIABLO, JNK and Caspases.[87] As such, activation of E2F1 can be lethal for neurons. Indeed, knockdown of, or interference with, E2F1-mediated transcription has provided a protective effect against such insults as dopamine-induced toxicity, serum and potassium deprivation and excitotoxicity in cerebellar granule neurons.[95,96] Furthermore, *in vivo* knockout of E2F1 is protective against MPTP toxicity in mice, a compound known to induce PD-related symptoms.[97] In line with this is the finding that in the substantia nigra, mid-frontal cortex and hippocampus of patients with PD, pRb phosphorylation is increased and is associated with Lewy Bodies in the substantia nigra.[98] NPM has thus far displayed a positive role in regulating E2F1-mediated transcriptional activity; however, this has been examined in proliferative cells. As E2F1 is negatively associated with neuronal viability, it is of interest to see if an NPM/E2F1 relationship not only occurs in post-mitotic neurons, but leads to neuronal death. It is possible that the outcome of their interaction is situation-dependent. NPM may indeed provide a protective effect for neurons, but in the face of oxidative stress, may facilitate DNA repair machinery through E2F1 that can ultimately lead to apoptosis.

Interestingly, other NPM cell cycle partners also display negative characteristics with regard to neurodegeneration. Inhibition of ROCK II either pharmacologically or by shRNA-mediated knockdown has provided a protective effect against the degeneration of dopaminergic neurons in models of PD[99] as well as amyloid-β production in a mouse model

of AD.[100] Similarly, inhibition of Plk1 has provided protection against β-amyloid-induced death in AD[101] and Plk2 can phosphorylate α-synuclein at Ser-129, a modification that is a hallmark of PD.[102] Further, GADD45α can mediate glutamate- and kainic-acid-induced oxidative toxicity in HT22 neuroblastoma cells and in the rat hippocampus, respectively, as part of the JNK-p53 signaling cascade.[103] However, it also protects dorsal root ganglion neurons after nerve injury.[104] As with E2F1, it remains to be determined if a functional relationship exists between NPM and these cell cycle related proteins in post-mitotic neurons. Furthermore, the effect of NPM's role with centrosomes in neurons requires elucidation. While yet to be examined, its inhibition of centrosome duplication would indicate a pro-survival role for neurons as entrance into the cell cycle leads to neuronal death. However, NPM's expression level might be critical for this regulatory role. As stated, the toxicity our lab has found from NPM overexpression into primary neuronal cultures is blocked by CDK inhibition, and specifically roscovitine, which is known to target CDK2. It is possible that the normal levels of endogenous NPM provides the necessary blockade of cell cycle induction, but increased levels from overexpression can override this and induce the cell to proliferate. More work remains, though, to delineate the role of NPM in centrosome biology in neurons.

Nucleolar stress and neurodegeneration

The role of the nucleolus in actively proliferative cells has been known to involve the regulation of ribosome biogenesis and cell growth. As such, it is important during neuronal development and neurite outgrowth. The nucleolus is also a critical regulator of the cell's response to stressors, such as oxidative stress and DNA damage, through p53 regulation. While less is known about nucleolar function in post-mitotic neurons, as well as its instability and the impact of nucleolar stress on the onset and progression of neurodegenerative diseases, there has been an upward momentum in recent years.

Changes to nucleoli and increases in nucleolar stress have been observed for many neurological diseases. AD and PD, as well as a mouse model of Rett syndrome, all display a reduction in nucleolar size.[105] In early stages of AD, patients display oxidized rRNA and decreased ribosomal activity, as seen by decreases in both rRNA and tRNA levels as well as hypermethylation of CpG islands of rDNA promoters.[106–108] Hypermethylation of the upstream control element (UCE) of the rRNA promoter also occurs in the R6/2 HD transgenic mouse model, and mutant huntingtin prevents nucleolin binding to the UCE, resulting in downregulation of pre-47S rRNA and p53-mediated cell death.[109] Upstream binding factor (UBF) is a transcription factor that is essential for rDNA transcription and can be regulated through acetylation by CREB-binding protein (CBP) and methylation by ESET/SETDB1. In R6/2 mice, mutant huntingtin sequesters CBP in nuclear aggregates, interfering with its targeting of UBF, thereby leading to a decrease in rRNA transcription.[110] Additionally, in the striatum of these mice, SETDB1 expression is increased and specifically interacts with and increases UBF methylation.[110]

Both *in vitro* and *in vivo* examination of nucleolar stress and neuronal dysfunction have focused on inhibition of rRNA transcription. Kalita et al. have reported that DNA damage in cortical neurons induced by camptothecin treatment inhibits RNA polymerase I

transcription, increases nucleolar stress and activates p53-mediated neuronal apoptosis.[111] This is further seen by shRNA-mediated knockdown of the RNA polymerase I co-factor, transcription initiation factor IA (TIF-IA). Similarly, Parlato et al. have shown that *in vivo* ablation of TIF-IA in neural progenitor cells results in p53 upregulation and massive apoptosis.[112] While this is also observed in the hippocampus, degeneration is prolonged over several months. A substantia nigra specific TIF-IA knockout also has increased p53 expression but shows decreased mTOR activity, mitochondrial dysfunction and increased oxidative stress, which are hallmarks of PD.[113] These mice further displayed the slower progressing degeneration as observed by Parlato et al. Interestingly, a similar knockout in R6/2 mice produced in medium spiny neurons of the striatum, the cell type affected in HD, displayed a pro-survival effect before degeneration through the inhibition of mTOR by p53-activated PTEN.[114] All of these reports observed a nucleoplasmic relocalization of NPM from nucleoli. This shuttling is an event usually seen with DNA damage, which might account for the increased p53 expression as NPM is known to stabilize p53 upon such an insult to the cell.[111–114] Unfortunately, however, this is the extent to which NPM has been examined with relation to nucleoli and neurons. Interestingly, when TIF-IA, and thus rRNA synthesis, is knocked out in the adult mouse, degeneration is a slower process that spans several months. While NPM is important for 28S maturation and the transport of ribosomal subunits to the cytoplasm in proliferative cells, it is possible that this role is less critical in mature neurons. NPM's main role in the neuronal nucleolus may instead be to act as a sensor for DNA damage and initiate mechanisms, such as p53 stabilization, to combat the problem. While it is clear that NPM's extrusion from the nucleolus in response to nucleolar stress can be harmful to the cell, more work is required to elucidate the downstream effect of this relocalization. Furthermore, it is also important to examine the effect, if any, of NPM relocalizing to the nucleoplasm in neurons under homeostatic conditions where DNA damage is not a factor.

Concluding thoughts

To date, NPM has been well-established in actively dividing cells as a protein that regulates key cellular processes, such as ribosome biogenesis and centrosome duplication, that are vital for a cell's ability to proliferate. As such, its expression is frequently altered in many tumors and cancers. Interestingly, NPM is highly abundant in post-mitotic neurons, but our knowledge of its role in these cells is greatly lacking. Furthermore, with the exception of a few studies centered on excitiotoxicity, where it has displayed a pro-survival role, NPM has thus far not been fully investigated in the context of neurodegeneration.

Owing to the diverse array of biological processes it helps to control, it is of course possible that functional differences exist for NPM in post-mitotic neurons. It is most likely that, similar to many other proteins, it can display dual roles. In normal, healthy neurons, NPM may function to regulate the cell's homeostatic state, thereby keeping it alive. For instance, as it is known to possess chaperone activity, NPM may help combat proteins that misfold and aggregate to become hallmarks of neurodegenerative diseases, like Aβ, α-synuclein and mutant huntingtin. However, in the face of insult, it may act as a sensor for certain stressors, such as oxidative and nucleolar stress, and help to coordinate a response that can lead to cell death. Many of its known interacting partners are important for cell growth and division, but

these interactions may only occur in a pro-proliferative manner, after the neuron has re-entered the cell cycle.

One caveat though is that NPM's expression level may be a critical factor in determining its function in neurons as both too little or too much can be harmful to the cell. This has indeed been found in our lab from work in primary neuronal cultures. Nevertheless, while much remains available for discovery, NPM is an imporant and exciting molecule with great potential in the fight against neurodegeneration.

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Table 1

The role of NPM in proliferative cells.

Interaction	Effect on/Function of NPM	Effect on the cell	Reference
Ribosome biogenesis			
TTF-1	Imports TTF-1 into the nucleolus	rRNA transcription	21
pre-47S rRNA	Cleaves ITS2 of 32s rRNA	Maturation of 28S rRNA	7, 8
CDK1, B23.2, NPM3	Phosphorylation by CDK1 and/or heterotrimerization with B23.2 or NPM2 inhibits NPM RNA binding activity	Inhibition of 28S maturation	11, 13
ARF	Sumoylation of NPM	NPM nucleolar localization and protection from caspase-3 cleavage	23, 24
		Inhibition of 28S maturation	
Senp3	Reverses ARF sumoylation	Maturation of 28S rRNA	24
PARP-1 and PARP-2	PARP-1, PARP-2 and NPM extruded from nucleolus together following inhibition of RNA Pol I	rRNA quality control	9
APE1/Ref1	Stimulates APE1 endonuclease activity on abasic dsDNA but reduces activity on ssRNA	rRNA quality control	8
40S and 60S ribosomal subunits	Transport and export of ribosomal subunits to cytoplasm	Ribosome production	3, 16, 17
CRM1 complex CK2	Phosphorylation by CK2 promotes dissociation of substrate bound to NPM	Inhibition of NPM chaperone activity	15
Centrosome duplication			
Centrosome	Bound to unduplicated centrosomes between pairs of centrioles	Inhibition of centrosome duplication and centriole pairing	4, 26, 27, 30
Cyclin E/CDK1	Phosphorylation by Cyclin E/CDK1 promotes dissociation of NPM from centrosomes	Centrosome duplication	26, 27
Crm1	Reassociates with centrosomes via Crm1	Inhibition of centrosome duplication	4, 26, 30
ROCK II	Interacts with ROCK II at centrosomes following Cyclin E/CDK2 phosphorylation	Increased ROCK II kinase activity	28
Oxidative stress and DNA damage			
p53	Stabilizes p53	Increased p53 transcriptional activity	35, 38, 39
	Inhibits p53	Protects against stress-induced apoptosis in normal and malignant hematopoietic cells	
p53 and ATR	Represses p53 and inhibits ATR phosphorylation of p53	Sets the threshold for p53 response to UV-induced stress	
MDM2	Inhibits MDM2 following UV-induced damage	Stabilizes p53	37
p21	Negatively regulates p21 ubiquitination	Stabilizes p21	40
ARF	Interacts with ARF in nucleoli	Stabilizes ARF	44, 45, 46, 49
		Inhibits ARF targeting of MDM2	
Akt	Phosphorylation by Akt following DNA damage disrupts interaction with ARF	Inhibition of MDM2 and stabilization of p53	49, 50
pRb and E2F1	Sumoylated NPM interacts with hyperphosphorylated pRb in nucleoli during late S-phase/early G_2	Increased E2F1 transcriptional activity	23, 54, 55
	Upon UV-induced damage, phosphory-		

Interaction	Effect on/Function of NPM	Effect on the cell	Reference
	lated NPM associates with pRb, blocking repression of E2F1		
GADD45α	Regulates nuclear import of GADD45α	Inhibition of G2/M transition of the cell cycle	43
MnSOD	Co-activator with NF-κB to induce expression of MnSOD following treatment with PMA and cytokines	Regulation of MnSOD expression	94
Regulation of apoptosis			
PI(3,4,5)P3	Inhibits CAD	Blocks DNA fragmentation	23, 56-58
Akt2	Nuclear Akt2 protects unsumoylated NPM from caspase-3 cleavage and dictates nucleolar localization	Enhanced cell survival Promotes tumorogenesis in human lung cancer cells	60, 61
MAPK/ERK	nPKC activation of MAPK/ERK signaling causes downregulation of NPM during differentiation	Differentiation of the cell	65
K-Ras	Interacts with GDP and GTP bound K-Ras at plasma membrane	Increased K-Ras stabilization and clustering leading to increased MAPK signaling	67
RelA	Relocalized to cytoplasm by nucleolar sequestered RelA	Decreased NF-kB transcriptional activity and increased apoptosis.	70, 73, 75
Bax	Colocalizes with Bax and p53 at mitochondrial membrane.	Induction of apoptosis	76-78
p53	Interferes with p53 mitochondrial localization	Inhibition of apoptosis	

Table 2

NPM in post-mitotic neurons

In vivo model	*In vitro* model	Impact on cell viability	Effect on NPM	Reference
Spinal cord injury				
Mice injected with recombinant human G-CSF following hemisection of the right hemicord	G-CSF treated neurons isolated from the spinal cords	Neuroprotection and locomoter recovery following G-CSF treatment	*In vivo* upregulation of NPM expression NPM inhibition partially blocked G-CSF neuroprotection	82
Excitotoxicity				
PS1 M146V mutant transgenic mice	Glutamate-induced excitiotoxicity of cortical neuronal cultures	Decreased survival rate	Decreased expression of NPM mRNA	84
NMDA-induced excitiotoxicity in the cerebral cortex of mice	PC12 cells	Protection against SIAH1-GAPDH induced death	NPM disrupts SIAH1-GAPDH complex	85
	NMDA-treated primary cortical neurons		Overexpression in cortical neurons and mice inhibited NMDA-induced toxicity by inhibiting SIAH1	
Kainic acid (KA)-induced excitiotoxicity in rats	KA-treated primary cortical neurons and SH-SY5Y cells	Decreased NPM shows increased p53 levels in KA-treated SH-SY5Y cells	Downregulation of NPM protein in degenerating neurons of the hippocampal CA1 region of KA-treated rats and KA-treated primary cortical neurons	86
	NE-4C cells	Apoptosis following NPM downregulation is p53-independent		
Nucleolar Stress				
	Cultured cortical neurons treated with camptothecin	Reduced rRNA transcription and p53-mediated apoptosis	Nucleoplasmic relocalization from nucleoli	111
TIF-IA knockout in neural progenitors and hippocampal neurons		Impaired nucleolar activity and increased p53 levels Rapid apoptosis in neural progenitors Prolonged degeneration in the hippocampus	Nucleoplasmic relocalization from nucleoli	112
Post-mortem brain sections of PD patients		Nucleolar damage in human PD brains	Nucleoplasmic relocalization from nucleoli	113
TIF-IA knockout in dopaminergic neurons of mice		TIF-IA knockout results in parkinsonism in mice		
Mice injected with MPTP		Increased p53 levels		
		Downregulation of mTOR activity		
TIF-IA knockout in medium spiny neurons of R6/2 mice		Late progressive striatal degeneration	Nucleoplasmic relocalization from nucleoli	114
		p53 prolongs survival through PTEN upregulation, inhibition of mTOR signaling and activation of autophagy		