THE REGULATION OF NEURONAL SURVIVAL THROUGH THE MODULATION OF ABERRANT CELL CYCLE RE-ENTRY

by

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This dissertation is dedicated to my parents, Dan and Debbie Pfister, my brother Todd, and Jake.

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The complex nature of the neuron is made that much greater knowing there are billions of them forming trillions of synaptic connections all while working in concert with billions of non-neuronal glial cells. To further complicate the highly intricate environment that forms the brain, upon maturation neurons become senescent, or post-mitotic, and will no longer regenerate. Nucleophosmin 1 (NPM1) is a highly abundant and ubiquitously expressed nucleolar phosphoprotein. While actively investigated for its role in the regulation of many cellular processes critical for proliferative cells, little is known about its role in the brain. The primary focus of this dissertation is to examine the effect of NPM1 on the regulation of neuronal viability. The dissertation is divided into the following four chapters:

I begin in chapter 1 by providing an overview of the mechanisms regulating a form of programmed cell death known as apoptosis or cell suicide. While there are many causes that can lead to the initiation of neuronal apoptosis, one well-accepted method is through a neuron's

aberrant attempt to divide. In order to understand how this may occur, this chapter provides an overview of the complex regulation of the cell cycle.

In chapter 2, I summarize what is currently known about the roles of NPM1 in actively dividing cells. Little has been published on neuronal NPM1. As such, this chapter concludes by describing these few studies, as well as what insights we can gain about its role in these cells from its functions in proliferative ones.

Chapter 3 describes a complex nature for neuronal NPM1. I show that while neurons require this protein for their normal healthy survival, increasing its expression is toxic. This toxicity is regulated by NPM1's ability to translocate to the cytoplasm and oligomerize. If restricted to the nucleus, which results in an inability to oligomerize, NPM1 no longer induces death and becomes fully protective against apoptosis.

Finally, in chapter 4 I extend previously published findings with new and unfinished data describing the neuroprotective roles for SIRT1 and SIRT5, two members of the Class III histone deacetylases (HDACs) that are collectively know as the sirtuins. I describe how SIRT1 is able to confer a protective effect in a deacetylase independent manner through a dependence on HDAC1. Lastly, I provide evidence that SIRT5 is able to protect neurons in a PKA-dependent manner.

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CHAPTER 1

THE REGULATION OF APOPTOSIS AND CELL PROLIFERATION

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II. ABSTRACT

The ability of a cell to remain healthy and live a long life is a tricky business that requires highly intricate interplay between multiple cellular processes. Dysfunction in just one aspect of the intracellular environment might be sustainable for a time, depending on the failure, but can ultimately lead to death by a process known as apoptosis, a type of programmed cell death. In almost every area of the human body the significance of cell death generally pales in comparison to that of neuronal death because while these other cells can regenerate, neurons never will. To further complicate matters for a neuron, any attempt to divide and replace a neighbor that has died will only lead to failure and apoptosis of itself. As the human brain contains billions of neurons that create trillions of connections to one another, losing individual neurons is only a minor inconvenience. However, excessive and progressive apoptotic death can lead to brain dysfunction, degeneration and, depending on the area sustaining the loss, the development of certain neurodegenerative diseases. These diseases reduce the quality of life for those that suffer from them as well as family members that struggle to care for such affected individuals. Since no cures exist and the only treatments available attempt to ease symptoms and reduce the speed at which neuronal numbers decline, the unfortunate consequence of developing a neurodegenerative disease is that it will ultimately result in death. The focus of this chapter is to provide an overview of how both apoptosis and cell proliferation are regulated.

III. APOPTOSIS, A FORM OF PROGRAMMED CELL DEATH

During all stages of its life cycle, a multicellular organism requires the ability to effectively clear out unwanted cells. Those that become damaged by external factors, such as by trauma, injury or viral infection, can become necrotic and die prematurely through unregulated autolysis. However, the death of cells during such times as development and the maintenance of homeostasis is carried out by one of two highly regulated processes, autophagy and apoptosis, that together are known as programmed cell death (PCD). Autophagy results in the degradation and recycling of intracellular cytoplasmic components and occurs through three pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy. The main pathway, known as macroautophagy, functions by engulfing excess, dysfunctional, or damaged proteins or organelles in an autophagasome, a double-membraned vesicle, that will then fuse with lysosomes thereby resulting in the degradation of its contents. Microautophagy, however, occurs by the lysosome directly engulfing cytoplasmic contents. Finally, chaperone-mediated autophagy is a complex process that requires a protein to contain a recognition site for the chaperone HSP70. An HSP70-containing complex will recognize the protein and transfer it to the lysosome where it will be unfolded and translocated across the lysosome membrane. Dysfunctions in autophagy can result in the build-up of these damaged and unwanted proteins, ultimately creating a harmful intracellular environment.

However, on the other hand, when a cell commits to die through apoptosis, also known as cell suicide, a highly regulated sequence of events is followed that ultimately leads to the cell's

engulfment and removal by phagocytes to protect against damage to surrounding cells. This cellular process is critical during all times of life. As an example, during embryonic development humans and many animals are born without webbed hands and feet due to the webbing between digits dying off by apoptosis. Also, during vertebrate development, approximately 50% of all neurons die by this process. Further, a frog is born without a tail due to the fact that these cells die during metamorphosis from a tadpole. It is estimated that for adults, roughly 50-70 billion cells die by apoptosis each day. Therefore, due to its necessity its proper regulation is vital to the body.

The two best-described mechanisms that lead to the initiation of apoptosis are the intrinsic and extrinsic pathways, which are regulated by intracellular signals or extracellular ligands binding to specific cell-surface receptors, respectively. While these pathways differ, they both converge to a series of shared morphological changes that result in cell death (1). This begins with the degradation of organelles and a rapid global mRNA decay, which is followed by shrinkage of the cell, chromatin condensation (pyknosis) and then blebbing of the plasma membrane. The nuclear envelope and then DNA become fragmented (karyorrhexis) and the nucleus is broken up into what is known as chromatin bodies. Finally, the cell is broken apart into smaller vesicles called apoptotic bodies that are phagocytosed.

Caspases

Independent of the apoptotic pathway that is activated, the death of the cell is controlled by a group of enzymes known as caspases (cysteine-aspartic proteases or cysteine-dependent aspartate directed proteases). These enzymes are named as such because they require cysteine in their active site and cleave after specific aspartate residues. To date, 13 caspases have been

identified. While caspase-1, -4, -5, -11, -12, and -14 are known as inflammatory caspases, the rest have roles in apoptosis and are subdivided into initiator caspases (-2, -8, -9, -10) and effector caspases (-3, -6, -7) (2). All caspases are synthesized as inactive pro-enzymes, or zymogens, and are post-translationally activated by dimerization through their pro-domains and cleavage into a large and small subunit. The pro-domain of initiator caspases consists of a caspase recruitment domain (CARD) and a death effector domain (DED), the later of which is important for interaction with other caspases. Following dimerization the initiator caspases will autoproteolytically cleave their pro-domains, heterodimerize their small and large subunits and then form heterotetramers (3). Now active initiator caspases can then cleave and activate the executioner caspases, which will in turn cleave their intracellular targets.

The Intrinsic Pathway

The intrinsic, or mitochondrial, pathway is induced by non-receptor mediated stimuli such as growth factor withdrawal, radiation, viral infection, and toxins. Initiation results in the permeabilization of the outer mitochondrial membrane by the formation of an ion channel known as the mitochondrial apoptosis-induced channel (MAC) by members of the Bcl-2 family of proteins and the subsequent release of Smac/DIABLO, HtrA2/Omi, and cytochrome *c* into the cytosol (3). Once released, Smac/DIABLO and HtrA2/Omi bind to and inhibit proteins known as IAPs (inhibitors of apoptosis proteins) that include XIAP, c-IAP1, Living and Survivin, whose function is to bind to and inhibit caspases. Cytochrome *c* however will interact with the C-terminal WD-40 domain of the adapter protein Apaf-1 (apoptotic protease activating factor-1) in a 1:1 ratio (4). The subsequent binding of ATP will allow Apaf-1 to oligomerize through its central nucleotide binding and oligomerization domain (NB-ARC/NOD) with seven other Apaf-

1/Cytochrome *c* molecules to create an active apoptososme (5). Simultaneously, pro-caspase 9 is then recruited to the N-terminal CARD domain of Apaf-1 leading to its cleavage to an active caspase-9 and subsequent activation of caspase-3 (5, 6). Cleaved (active) caspase-3 is then free to target its downstream effectors such as cleaving, and thus inhibiting, the inhibitor of caspase activated DNase (ICAD), allowing caspase-activated DNase (CAD) to induce DNA fragmentation (7).

The Bcl-2 Family

Perhaps the most important regulation of mitochondrial-mediated apoptosis occurs through a group of 25 proteins that together are known as the Bcl-2 family (8). These proteins can be either anti- or pro-apoptotic and function by forming homo- or heterodimers to control mitochondrial outer membrane permeabilization (MOMP) and thus cytochrome *c* release. All members of the Bcl-2 family share at least one of four domains of homology, termed Bcl-2 homology (BH) domains, which are referred to as BH1-4. Furthermore, many members also possess a transmembrane domain (TM) at their C-terminal responsible for insertion into the outer mitochondrial membrane. With the exception of one member, MCL-1, that lacks the N-terminal BH4 domain (9), the anti-apoptotic Bcl-2 proteins contain all four BH domains as well as a TM (10). Members of this class of Bcl-2 proteins include: Bcl-2, Bcl-XL, Bcl-w, MCL-1 and A1 (3). The pro-apoptotic Bcl-2 members are subdivided into two classes, the effectors which are multi-domain and the BH3-only proteins that lack all domains but BH3. For all pro-apoptotic members, the BH3 domain is necessary for dimerization with other members of the Bcl-2 family. Among the effectors, which all possess a TM, Bax, Bak, and Bok all have BH1-3 (3), whereas Diva contains BH1-4 (11) and Bcl-Xs contains BH3-4 (12). The BH3-only members include:

Bad, Bid, Bik, Bim, Bmf, Hrk, Noxa and Puma, and of these Bim, Bik and Hrk also contain a TM (13). Due to the presence of the TM, the effector Bcl-2 proteins are responsible for MOMP whereas the BH3-only members act to relay the apoptotic signal.

Two models have been proposed to described how these effectors, most notably Bax and Bak, lead to the MOMP and the release of cytochrome *c* and other pro-apoptotic molecules (3, 14). First, in what has been termed the indirect activator or neutralization model, the effectors are constitutively active and kept inhibited through direct interaction with anti-apoptotic Bcl-2 proteins (3). When apoptosis is induced, the BH3-only proteins compete for binding to these anti-apoptotic Bcl-2 members, thus freeing Bax and Bak to form the MAC. Conversely, the direct activator-derepressor model states that the effectors are instead activated through interaction with a subset of BH3-only members referred to as the direct activators. Simultaneously, another subset of BH3-only proteins, known as sensitizers, bind to anti-apoptotic Bcl-2 members and block their inhibition of the effectors (14). While each model is just as likely on its own, it is probable that both, or at least aspects of both, are true.

The Extrinsic Pathway

While the intrinsic pathway involves intracellular cues, the extrinsic, or death receptor pathway, is mediated by extracellular signals through transmembrane death receptors from the TNF (tumor necrosis factor) receptors family. This overall pathway can be executed by two characterized paths, FasL/FasR or TNF-α/TNFR1 (15). The Fas ligand (FasL), a transmembrane protein located on an adjacent cell, binds as a homotrimer to the transmembrane Fas receptor (FasR, also known as Apo-1 or CD95) containing an intracellular death domain (DD). This, in turn, causes

receptor oligomerization, aggregation of the DD and internalization of the receptor complex by endocytosis. The adaptor molecule, Fas-associated protein with death domain (FADD), is then allowed to bind to the complex through its own DD. FADD additionally contains a DED that aids in the binding to the DED of initiator caspases procaspase-8 (also known as FLICE) or procaspase-10, forming the death-inducing signaling complex (DISC). These initiator caspases then self-cleave to activate as previously described, are released from the DISC and cleave downstream effector caspases. While the Bcl-2 proteins are generally known as mediators of the intrinsic pathway, crosstalk with the extrinsic pathway has been shown as active caspase-8 can cleave the BH3-only protein Bid to a truncated form known as tBid to mediate cytochrome *c* release (16).

The TNF-α-mediated extrinsic pathway functions in a similar manner to Fas-signaling. Homotimeric TNF-α binds to its receptor (TNFR1) resulting in receptor trimerization and internalization into the cell. This activation results in the recruitment of the TNFR1-associated death domain protein (TRADD) to the receptor, which in turn serves as an adaptor to three other proteins, receptor-interacting protein 1 (RIP1), Fas-associated death domain protein (FADD), and TNF-receptor-associated factor 2 (TRAF2) (15). Just as in Fas signaling, FADD recruits pro-caspase-8 to initiate apoptosis. RIP1 (also known as RIPK) can recruit the death adapter protein RAIDD (also known as CRADD), which will in turn recruit procaspase-2 (17). Interestingly, TRAF2 and RIP1 can also activate NF-κB, a complex with pro-survival qualities (18). Similar to the Fas-regulated pathway, crosstalk with the intrinsic signaling has been shown through TNF-α whereby TRAF2 induces c-Jun N-terminal kinase (JNK) activation, a well-established pro-apoptotic molecule, that in turn cleaves Bid to jBid leading to MOMP (19).

While apoptotic signaling through TRAF2 is normally inhibited by the IAP, c-IAP1, the release of Smac/DIABLO from mitochondria abolishes this inhibition leading to caspase-8 activation (19).

Caspase-Independent Apoptosis

While caspase-mediated apoptosis is the predominate and best studied method of programmed cell death, caspase-independent mechanisms additionally occur. Following MOMP, while mediators of the intrinsic pathway are released into the cytosol, other factors, including apoptosis inducing factor (AIF) and endonuclease G (EndoG), are additionally release that can induce apoptosis. AIF is a mitochondrial flavoprotein that localizes to the intermembrane space where it has a role in oxidative phosphorylation by regulating complex I (20). Once released from the mitochondria, AIF translocates to the nucleus and induces chromatin condensation and DNA fragmentation (21). EndoG is also localized to the intermembrane space within the mitochondria. It has been proposed to play a role in mitochondrial DNA replication by generating RNA primers required by DNA polymerase γ (22). Further, it also contains RNase and RNase H activity (22). Following release from mitochondria, Endo G can translocated to the nucleus and cleave DNA at double-stranded $(dG)_n \bullet (dC)_n$ and at single-stranded $(dC)_n$ tracts (22). Finally, as previously discussed HtrA2/Omi is released from mitochondria during apoptosis and targets IAPs causing the release of caspases. In a method that can be characterized as caspase-independent, HtrA2/Omi is able to recruit IAPs through its first four N-terminal amino acids (AVPS) which constitutes and IAP-binding motif. Due to its serine protease activity, it is able to

cleave the IAPs it comes into contact with thereby reducing their presence in the cell and ability to inhibit caspase activation (23).

IV. CELL CYCLE REGULATION

A cell's ability to successfully divide is necessary for proper development and homeostatic maintenance of the body. This is maintained by a highly regulated process known as the cell cycle. One caveat to the central nervous system though is that once neurons fully differentiate they become what is referred to as post-mitotic and enter a state of senescence where they will spend to rest of their days. While the induction of apoptosis in neurons can be initiated through a multitude of factors, one well-excepted theory that emerged in the mid-1990s for how this can occur is through aberrant cell cycle re-entry (24). Even though certain areas of the brain, such as the hippocampus, have exhibited neurogenesis into and through adulthood (25), in general when a neuron attempts to exit their senescent state and divide, they are unfortunately rewarded with death by cell suicide (apoptosis). Indeed, important mediators of cell cycle re-entry are upregulated in the brains and post-mortem tissues of patients affected by neurodegenerative diseases such as Alzheimer's Disease, Parkinson's Disease, and amyotrophic lateral sclerosis (24, 26–29). Furthermore neuronal loss in the cerebral cortex of Down syndrome patients has correlated with mitogenic signal upregulation (30) and evidence of cell cycle re-entry has been found following stroke and traumatic brain injury (24). While ample evidence of activation and/or upregulation of cell cycle machinery exists following neuronal death, it is relatively unclear if their expression is a cause or effect (24). Thus, in order to elucidate how this death may occur, it is important to understand how the process of cell division, or the cell cycle, is regulated.

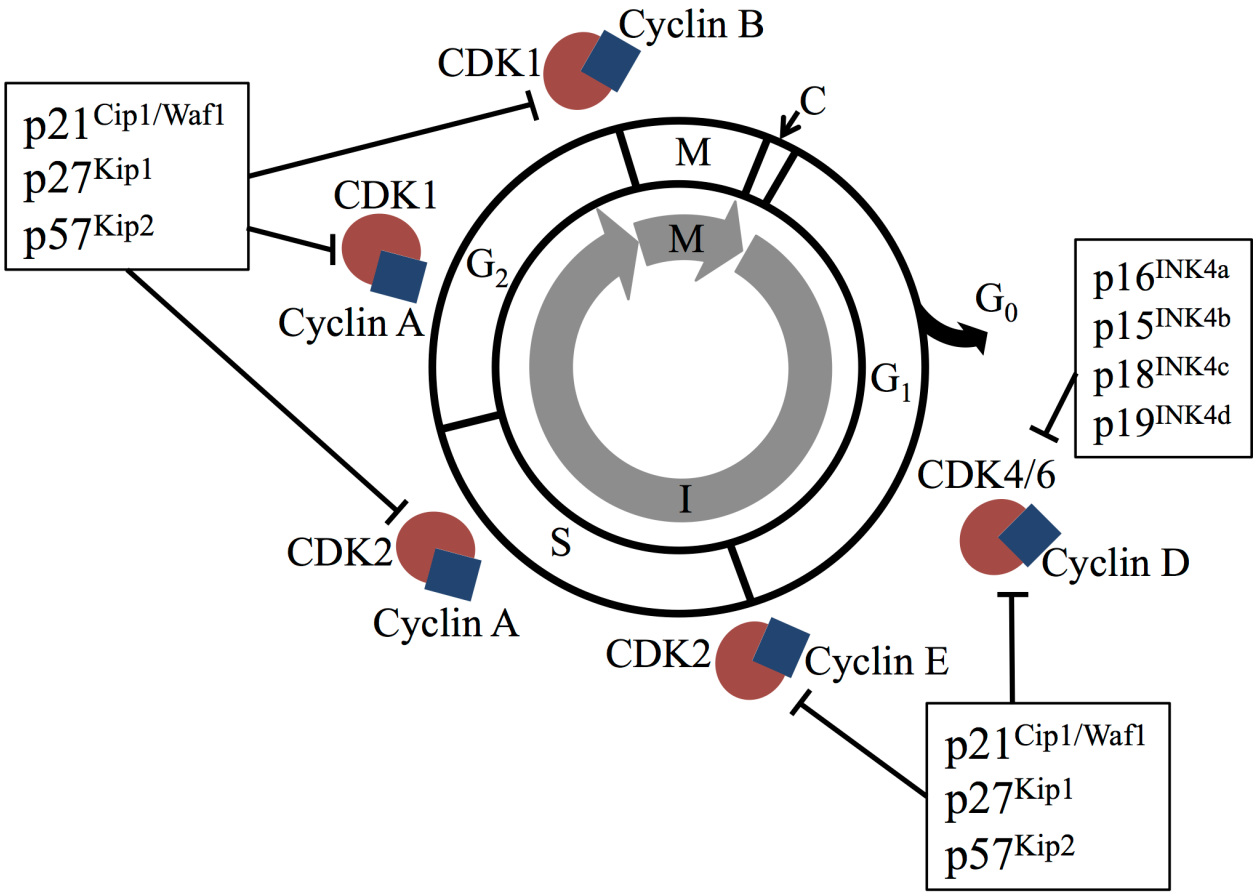

Figure 1.1. Schematic of the cell cycle. The cell cycle is divided into two main phases: interphase (I) and mitotic or M-phase (M). Interphase consists of Gap 1 (G_1), S-phase (S) and Gap 2 (G_2). The M-phase is defined by mitosis (M) and cytokinesis (C). After cell division in cytokinesis, the cell grows in G_1 and can enter a senescent state (G_0). Each phase is regulated by a cyclin/CDK complex that can be inhibited by two families of proteins known as cyclin-dependent kinase inhibitor proteins (CKI). The INK4 family consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$ that target CDK4 and CDK6 and the Cip/Kip1 family that targets all cyclin/CDK complexes contains $p21^{Cip1/Waf1}$, $p27^{Kip1}$ and $p57^{Kip2}$.

As shown in figure 1.1, the cell cycle is divided into two main phases, interphase and the mitotic (M) phase. Early into interphase there is a period known as quiescence, also referred to as resting phase, Gap 0 (G_0) and post-mitotic, and is a characterized by the cell entering a state of senescence. Cells can remain in G_0 for long periods of time or, as in the case of neurons,

indefinitely. When in preparation for division, the cell will leave G_0 and enter into interphase. The first stage of interphase, Gap 1 (G_1), is a time defined by extensive cell growth and an increase in intracellular proteins and organelles. From here the cycle moves into S-phase (synthesis phases) where the cell's DNA is replicated to produce a copy, or chromatid. The original chromosome and its copy, referred to as sister chromatids, are bound together by a part of the chromosome called the centromere that functions as a site for assembly of the kinetochore. The kinetochore is a large and complex protein structure that assembles on each sister chromatid, faces towards opposite poles of the cell and functions as the site for spindle fiber attachment. The centromere is also a region acted upon by the cohesin, a complex consisting of four subunits that forms a ring structure around the sister chromatids and holds them together. It is also during this time where there is duplication of the centrosome, which functions as the major microtubule-organizing center (MTOC). Centrosomes are composed of two orthogonally oriented centrioles that are surrounded by pericentriolar material (PCM). This PCM contains a group of proteins that are responsible for microtubule nucleation and anchoring. The final stage of interphase, Gap 2 (G_2) is a period of increased cell growth and protein synthesis that is performed in preparation for cell division. When the cell is ready to divide it halts its growth and enters the mitotic phase, or M-phase, which consists of mitosis followed by cytokinesis where cell division into two daughter cells occurs.

Mitosis is a complex and highly regulated process that is itself broken up into five distinct phases: prophase, prometaphase, metaphase, anaphase and telephase. During prophase, gene transcription is halted, the nucleolus disappears, chromosome condensation occurs, and the mitotic spindles begin to form between the pairs of centrosomes. With the help of molecular

motors, the centrosomes are pushed towards opposite ends of the cell nucleus. Early on in prometaphase, the nuclear envelope begins to break down due to nuclear lamin (lamin A, B, and C) phosphorylation by mitosis-promoting factor (MPF) (31). The MPF additionally phosphorylates myosin-II light chain to prevent premature cytokinesis (32). With access to the chromosomes, kinetochore microtubules from a fully developed mitotic spindle attached to polar opposite kinetochores with the help the cohesin complex. The kinetochores then use the motor proteins in their structure to move each chromatid towards its respective centrosome. As mitosis enters metaphase, due to microtubule polymerization and depolymerization, the centrosomes begin pulling the chromosomes towards their opposite ends of the cell forcing them line up on the metaphase plate. The onset of anaphase begins with the cleavage and dissociation of the cohesins from centromeres thereby releasing the sister chromatids from one another, which thus forms two identical daughter chromosomes. Cleavage of the cohesins occurs by the protease, separase, which is normally inhibited by the chaperone securin (33). At the beginning of anaphase, the E3-ubiquitin ligase, anaphase promoting complex, or cyclosome (APC/C), targets securin for degradation which then frees separase (34). As the cell moves through anaphase, the kinetochore microtubules begin to depolymerize and pull the two new chromosomes towards opposite poles while non-kinetochore or polar microtubules push against each other, thereby elongating the cell. The final phase of mitosis, telophase, is marked by a continued elongation of the cell, the formation of new nuclear envelopes around each set of daughter chromosomes due to dephosophorylation of nuclear lamins, and the reappearance of nucleoli. With mitosis complete, a contractile ring made of actin and dephosphorylated myosin II forms at the location of the metaphase plate leading to the formation of a cleavage furrow. The cleavage furrow

continues to contracts until the parent cell eventually split into two daughter cells. The entire cell cycle process through one round of division is finished in roughly 24 hours. The cell spends ~90% of its time in interphase with ~10 hours in G_1, ~5-6 hours in S-phase, and ~3-4 hours in G_2. Mitosis however only takes about 2 hours to complete.

Cyclins, CDKs, and CDK Regulation

Progression through the cell cycle is governed by complexes between two groups of proteins, cyclins and the serine/threonine cyclin-dependent kinases (CDKs). While CDK expression is relatively constant their activity is regulated post-translationally by binding with cyclins, whose expression fluctuates (Fig. 1.2), and subsequent phosphorylation. As a result, proper progression through the different phases of the cell cycle is controlled by different cyclin/CDK complexes (Fig 1.1).

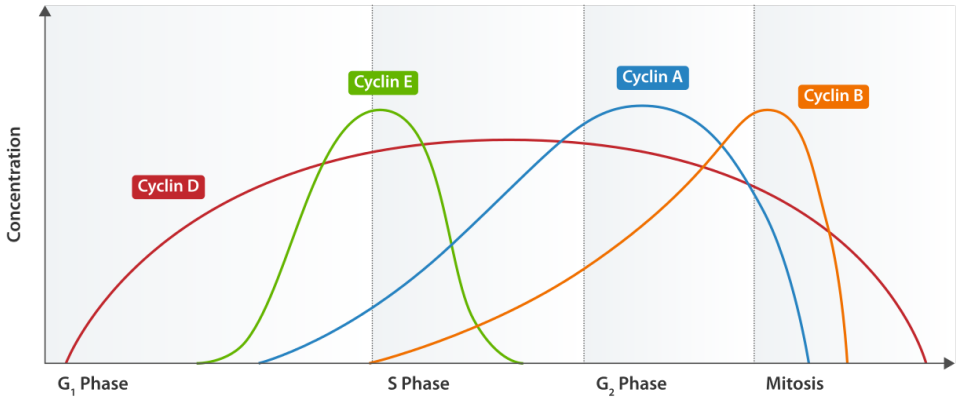

Figure 1.2. Cyclin expression through the cell cycle.

While humans express 15 different families of cyclins, comprising up to roughly 30 individual members in total, only five families regulate the cell cycle (35). These cyclins can roughly be

categorized into two main groups, those that regulate G_1 as well as the G_1/S transition, and thus the entry into the cell cycle, and those that regulate the G2/M transition into mitosis. Exit from G_0 and entrance into G_1 is control by the cyclin C/CDK3 complex (table 1.1) phosphorylation of retinoblastoma (Rb) and the resulting activation of members of the E2F family of transcription factors (36). As shown by figure 1.2 and table 1.1, the G_1 phase is largely regulated by Cyclin D. Humans express three known cyclin D proteins, termed cyclin D1-3 that form complexes with CDK4/CDK6. Their expression is regulated by mitogen receptors and Ras/Raf/ERK/MAPK signaling through the Myc and AP-1 transcription factors as well as the β-catenin-Tcf/LEF signaling pathway (37). In response to integrin, the Rho GTPases and FAK can additionally activate cyclin D (37). This in turn results in an increase in E2F activation leading to the

Table 1.1. Cyclin/CDK complexes that regulated cell cycle progression

Phase	Cyclin	CDK
G_0/G_1	C	CDK3
G_1	D	CDK4, CDK6
G_1/S	E	CDK2
S/G_2	A	CDK2, CDK1
G_2/M	B	CDK1

expression of cyclin E, which is expressed as two proteins, cyclin E1 and E2, and the subsequent activation of CDK2 (Fig1.2 and table 1.1). Progression into S-phase and through G_2 is governed by cyclin A (table 1.1). Humans expression two distinct cyclin A proteins; cyclin A1 is the embryonic-specific form of cyclin A and cyclin A2 is the somatic form (38). Cyclin A is the

only cyclin that functions across multiple phases of the cell cycle, thereby activating two different CDKs; CDK2 in S-phase and CDK1 through G_2 (Fig. 1.2 and table 1.1). Finally, as the cell nears mitosis, the expression of cyclin B, also known as the mitotic cyclin, is turned on to activate CDK1 (Fig. 1.2 and table 1.1). This cyclin B/CDK1 complex is known as the MPF (maturation-promoting factor, mitosis-promoting factor or M-Phase-promoting factor). Three cyclin B proteins, cyclin B1-3, have been discovered and while they are important for entry into mitosis, their rapid degradation is necessary for mitotic exit.

As described above, CDK activity is regulated post-translationally through cyclin binding and phosphorylation. As with all kinases, CDKs contain an ATP-binding site, or cleft, necessary for their activity. However, CDKs have a modified site that is blocked by a flexible loop known as the T-loop. Cyclin binding causes a conformational change of two alpha helices, L12 and PSTAIRE, in the CDK thereby permitting ATP binding (39). L12, which comes before the T-loop moves out, becomes a beta strand, and causes a rearranging of the T-loop (39). This then allows the PSTAIRE to move in; glutamate 51 within the helix interacts with lysine 33 and aspartate 145 changes position (39). These three residues then allow for effective ATP binding. Following cyclin interaction, the CDK-activating kinase (CAK), a trimeric protein complex consisting of CDK7, cyclin H and Mat1, phosphorylates the CDK rendering the cyclin/CDK complex active to target downstream effectors (40).

CDK Inhibition

While CDK activity is regulated through cyclin binding and CAK phosphorylation, they can also be inhibited through phosphorylation and the binding of proteins known and cyclin-dependent kinase inhibitors (CKIs). Two CKI families have been identified; the INK4 gene family, which

consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, and the Cip/Kip family whose members include $p21^{Cip1/Waf1}$, $p27^{Kip1}$ and $p57^{Kip2}$.

The sole targets of the INK4 family are CDK4 and CDK6 and function by interfering with cyclin D binding and thus CDK activation. All four are highly similar, or redundant, and are characterized by four ankryin repeats. The INK4a/ARF/INKFb locus, a 35 kb region on chromosome 9p21 encodes two of the four members, $p16^{INK4a}$ and $p15^{INK4b}$, as well as another tumor suppressor protein, alternate reading frame, or ARF. While $p15^{INK4b}$ has its own open reading frame producing a distinct protein, $p16^{INK4a}$ and ARF reading occurs down stream resulting in a different first exon with shared exons 2 and 3 that do not share amino acid homology. Knockout mice of all three are more prone to developing tumors but individual knockouts are less prone to tumor formation than a mouse containing both $p16^{INK4a}$ and ARF knocked out (41). Further, transgenic mice carrying an entire copy of the INK4a/ARF/INK4b locus show resistance to *in vitro* immortalization (42). As both $p16^{INK4a}$ and ARF expression normally increases with age, these mice showed a normal aging pattern (42). While $p16^{INK4a}$ and $p15^{INK4b}$ are CKIs, ARF functions through regulating p53 activation by targeting its inhibitor MDM2. ARF will be discussed in greater detail in chapter 2. Due to the importance of this locus in the inhibition of proliferation, deletions interfering with production of the three proteins is one of the most frequent events in human cancers (43). The gene locus for $p18^{INK4c}$ is located at the chromosome location, 1p32. While less is known about this CKI, and mutations in the locus are rarely seen with human cancers, it has been found silenced by promoter methylation in Hodgkin lymphoma (44) and medulloblastoma (45). It has been further found as a target of protein kinase c (PKC) where PKC can promote cancer cell growth by downregulating $p18^{INK4c}$

independent of AP-1 activation (46). Finally the locus for $p19^{INK4d}$ is located at 19p13. This CKI might not actually be tumor suppressor as knockout mice are not more susceptible to tumor formation than wild-type mice. However they do display increased germ cell apoptosis and testicular atrophy while still remaining fertile (47, 48).

The Cip/Kip family, on the other hand, is more versatile as they are broad spectrum and can target cyclin D/, E/, A/ and B/CDK complexes. Further, while the INK4 family interacts with the CDK to prevent cyclin binding, the Cip/Kip family can interact with both cyclins and CDKs through a shared N-terminal domain within each Cip/Kip family member. Inhibition of CDKs by this family occurs by blocking the substrate interaction domain within the cyclin and then blocking ATP binding to the CDK by inserting itself into the catalytic cleft (49). However, these proteins can also regulate cell cycle through interaction with non-CDK substrates.

While $p21^{Cip1/Waf1}$, $p27^{Kip1}$, and $p57^{Kip2}$ all function to inhibit cell proliferation and hence can act as tumor suppressors, the expression of each is initiated by different means. Perhaps the most well described transcriptional activator of $p21^{Cip1/Waf1}$ is the heavily investigated p53 tumor suppressor protein that functions as a critical regulator of the G_1/S transition by regulating the response to DNA damage and in turn apoptosis (50). As expected, mice lacking $p21^{Cip1/Waf1}$, while developing normally, exhibit defective G_1/S control due DNA-damage (51). $p27^{Kip1}$ expression is increased in quiescent states and during mitogen-depletion (52, 53). It has also been shown as rapidly degraded follow cell cycle entry (52, 53). Mice lacking $p27^{Kip1}$ show gigantism due to excessive proliferation, multiple organ hyperplasia, and female sterility (54). Contrary to these two, $p57^{Kip2}$ has been implicated as the only CKI important for regulation of embryonic development. Indeed, $p57^{Kip2}$ knockout mice die at birth due to multiple

developmental abnormalities including hyperplasia and delayed differentiation (55, 56). Further evidence of this role is found by its expression being regulated by many well-known factors important for embryogenesis such as Notch/Hes1, BMP-2 and -6, MyoD and p73 (57–60). CKI function and the ability to bind their targets can be regulated through both phosphorylation and protein-protein interaction. As an example, during S-phase, $p21^{Cip1/Waf1}$ becomes nuclear and is hyperphosphorylated by CDK2 at Thr-57 and then associates with Ser-126 phosphorylated cyclin B1 prior to CDK1 dephosphorylation at Tyr-15 by Cdc25 (61). While this interaction is poor, it is greatly increased following phosphorylation of CDK1 at Thr-161 which in turn leads to CDK activation and passing the G_2/M transition (61). Interference of this $p21^{Cip1/Waf1}$ phosphorylation through mutation or by knocking down the protein delays cyclin B1/CDK1 activity (61). Additionally, phosphorylation at Ser-145 by PKA, Ser-146 by PKC or Ser-160 by PKC prevented $p21^{Cip1/Waf1}$ from interacting with PCNA (proliferating cell nuclear antigen), which in turn blocked processive DNA synthesis (62, 63). Further, phosphorylation of $p27^{Kip1}$ on Thr-74, Thr-88 and/ or Thr-89 by Src, Lyn, or Abl, decreased its inhibitory effect on CDK2 complexes, however also increased its affinity for CDK4 (64–66). However, cyclin E/CDK2 can also phosphorylate $p27^{Kip1}$ at Thr187, leading to its ubiquitination and degradation thus promoting cyclin A production and S-phase entry (67). This degradation requires formation of a stable trimeric complex between $p27^{Kip1}$, cyclin E and CDK2 (68). While phosphorylation can directly interfere with substrate interaction as just described, it can also do so by altering CKI subcellular localization. PKB/Akt phosphorylation of $p21^{Cip1/Waf1}$ at Thr-145 blocks its interaction with importin and PKC phosphorylation of Ser-153 blocks the calmodulin binding site in $p21^{Cip1/Waf1}$ (69, 70). As a result both of these modifications prevent $p21^{Cip1/Waf1}$'s nuclear

import. Similarly, phosphorylation of $p27^{Kip1}$ at Ser-10 has resulted in its cytoplasmic localization. In quiescent cells, potential targeting by Mirk/Dirk promotes a binding site in $p27^{Kip1}$ for the CRM1/exportin1 complex, while in proliferative cells stathmin, PKB/Akt and ERK2 have been implicated in this export (52, 71–73). Further, the sequestering of $p27^{Kip1}$ in the cytoplasm due to interaction with 14-3-3, and thus interfering with its ability to interact with importin-α, has been shown by its phosphorylation at Thr-157 by PKB/Akt or Thr-198 by PKB/Akt or $p90^{RSK}$(74–76).

While predominately studied for their roles in CDK inhibition and cell cycle control, the Cip/Kip proteins also have roles in regulating apoptosis, transcription, and cytoskeletal dynamics. $p57^{Kip2}$ has shown both anti-and pro-apoptotic qualities. Following staurosporine treatment, $p57^{Kip2}$ promoted death via translocation to the mitochondria and favoring Bax activation (77), however it can also inhibit JNK1/SAPK-induced apoptosis through direct interaction and inhibition of JNK1 kinase activity (78). $p21^{Cip/Waf1}$ on the other hand has exhibited a more protective role. Through its N-terminus, $p21^{Cip/Waf1}$ can bind to pro-caspase-3 interfering with its cleavage and activation via Fas-mediated signaling (79). Further, $p21^{Cip/Waf1}$ can inhibit both JNK- and p38-mediated apoptosis through direct interaction with JNK as well as by blockage of the upstream activator of both proteins, ASK1/MEKK5 (80, 81). Cip/Kip regulation of transcription can occur either directly through cyclin/CDK complexes or indirectly. For instance, since all three Cip/Kip proteins block CDK4/6 activation, they thereby prevent phosphorylation of Rb family members, which in turn inhibits the activation of the E2F transcription factors as well as their downstream transcriptional targets. Through direct interaction, $p57^{Kip2}$ can also negatively regulate E2F1-mediated transcription and in turn RNA polymerase II C-terminal domain phosphorylation

(CTD) (82, 83). It has also been found to interact with MyoD, preventing MyoD degradation and inhibition of its transcriptional activity (84). Further, $p57^{Kip2}$ can directly interact with the pro-neuronal basic helix-loop-helix (bHLH) factor, Mash1, repressing its activity and neuronal differentiation of stems cells following mitogen withdrawal (85). Also through direct interaction, $p21^{Cip/Waf1}$ can inhibit the transcriptional activity of E2F1, c-Myc and STAT3 as well as derepress target genes for the p300/CBP complex (86–89). Finally, the Cip/Kip family can regulate cytoskeletal dynamics through the inhibition of the Rho/ROCK/LIMK/Cofilin signaling pathway. This pathway is initiated at the plasma membrane by a member of the Rho family of GTPases, RhoA, which activates RhoA kinase (ROCK). ROCK in turn activates LIM-kinase (LIMK), which then phosphorylates cofilin, the actin-polymerization factor, leading to actin cytoskeletal reorganization and the formation of stress fibers. Alternatively, another Rho GTPase, Rac1, activates its effector, p21-activated kinase (PAK) that leads to lamellipodia formation. $p57^{Kip2}$ can interact with LIMK1 and lead to its sequestration in the nucleus and a loss of stress fibers (90). Interestingly, $p57^{Kip2}$ knockdown in mice showed a delay in the migration of neurons in the cortical plate but did not effect neuronal differentiation (91). $p27^{Kip1}$ knockdown also showed an inhibition of neuronal migration into the cortical plate as well as the intermediate zone (91). However, in this same study, knockdown of $p27^{Kip1}$ resulted in increased $p57^{Kip2}$ mRNA levels (91). Interestingly, it can also stabilize Neurogenin-2 and promote differentiation of neuronal progenitor cells in the cortex (92). Fibroblasts cultured from a $p27^{Kip1}$ knockout mouse showed an increase in the numbers of stress fibers and focal adhesions as well as increased RhoA activity resulting in impaired motility that could be restored following ROCK inhibition (93). p27Kip1 was found to physically interact with RhoA, preventing binding to its

guanine nucleotide exchange factor (GEF) and thus blocking its activation (93). Cytoplasmic $p21^{Cip1/Waf1}$ can also interact with and inhibit ROCK1 and promote neurite extension in N1E-115 neuroblastoma cells as well as hippocampal neurons (94). Interestingly, RhoA itself can negatively regulate the expression of $p27^{Kip1}$ and $p21^{Cip1/Waf1}$, potentially created a negative feedback loop (95, 96). Thus evidence appears to indicate that while the Cip/Kip family of CKIs are not only important for regulating cell cycle progression, they are critical mediators in the migration of neurons during neuronal development through their effects on cytoskeletal dynamics. However, if their regulation of these Rho pathways has an effect on the viability of post-mitotic neurons remains unclear.

Cell Cycle Checkpoints

Within the cell cycle exists three points, referred to as checkpoints, where the cell halts proliferation and determines if conditions are favorable to continue. The first, known as the G_1 checkpoint or restriction point, is located just before the S-transition and acts as a point at which the cell decides if it can commit cell cycle entry. If proper conditions are not met, the cell will re-enter G_0 where it remains until allowed to continue. This checkpoint is maintained mainly through regulating the activity of the E2F transcription factors (E2F1-5) by three members of the Rb family of proteins, Rb, p107, and p130 (97). Rb binds to and inhibits E2F1-3, the mechanism of which is described in chapter 2, and p107 and p130 bind E2F4 and E2F5, which all block E2F's ability to transcriptionally activate G_1-to-S promoting factors. As the cell decides to leave G_0, cyclin D, which has a high turnover rate and regulated by mitogenic signaling, is activated and leads to CDK4/6 activation. Cyclin D/CDK4/6 complexes then phosphorylate Rb, releasing its hold on E2F thereby allowing transcriptional activation of Cyclins E and A that in turn

activate CDK2 into S-phase and through G_2, respectively. Rb phosphorylation and its inhibition is increased by CDK2 until it is dephosphorylated into a hypophosphorylated active and E2F-suppressing state by protein phosphatase 1 (PP1) during the mitotic exit (98). It is also during the restriction point that the cell checks for any instance of DNA damage. If found, the serine/threonine kinases, ATM and ATR, will phosphorylate Chk2 (99) and Chk1 (100), respectively, which can halt the cell cycle by two mechanisms. In one instance, Chk2 can phosphorylate the phosphatase Cdc25A, an activator of the Cyclin E/CDK2 complex, leading to its targeting for ubiquitination and degradation (99). On the other hand, Chk2 and Chk1 can phosphorylate and stabilize p53, which in turn transcriptionally activates $p21^{Cip1/Waf1}$ and thus CDK inhibition (101–103). However, p53 can also downregulate Chk1 in a $p21^{Cip1/Waf1}$- and Rb-dependent manner (104).

The second checkpoint encountered while progressing through the cell cycle is the G_2 or G_2-M DNA damage checkpoint, in which the cell must check for DNA damage following duplication during S-phase. As described, this G_2-to-M transition is governed by the Cyclin B/CDK1 complex. Similar to CDK2, activity of CDK1 is regulated by its respective cyclin and Cdc25. During G_2, CDK1 is kept inhibited by phosphorylation at Tyr-15 by a kinase known as Wee1 (105). Prior to mitosis, the kinase Plk1 phosphorylates Wee1, thereby targeting it for degradation, as well as Cdc25, leading to its activation (106). Cdc25 can in turn dephosphorylate CDK1 (107). This dual activity by Plk1 coupled with increased cyclin B expression results in MPF activation and entry into mitosis. If the cell cycle must be halted at this checkpoint due to DNA damage, the cell responds by a similar mechanism seen during the G_1 checkpoint; ATM/ATR activate Chk2/1, which deactivates Cdc25 and also activates/stabilizes p53. p53 in

turn upregulates 14-3-3 proteins that sequester Cdc25 in the cytoplasm, as well as $p21^{Cip1/Waf1}$, which will inhibit CDK1 activity.

The final checkpoint, termed the mitotic checkpoint, spindle checkpoint or spindle assembly checkpoint (SAC), occurs during mitosis and functions by preventing anaphase entry until the spindle has stably attached to the opposite polar kinetochores. Properly halting mitosis at the SAC is a complex process that is dependent on the inhibition of the APC/C through sequestration of Cdc20, a protein required for APC activation. Each kinetochore is a large complex composed of at least 80 different proteins and consists of an inner kinetochore responsible for centromere binding and an outer region that contains microtubule-binding complexes (108). This outer region contains three complexes, KNL1, Mis12, and Ndc80 that together comprise what is referred to as the KMN network (108). The Ndc80 complex, which is important for load-bearing attachments to microtubules, recruits the RZZ complex that is necessary for the recruitment of the dynein/dynactin motor complex (109, 110). The first protein important in the assembly of the SAC, a serine/threonine kinase called Bub1, binds to the Ndc80 early on in mitosis and functions to link the SAC to the kinetochores, as it is required for the recruitment of all downstream components (111, 112). A second protein, Bub3, also binds this region, possibly in complex with Bub1, and provides a binding site for BubR1. While the mechanism is not fully understood, it is known that KNL1 is required for kinetochore targeting of Bub1, Bub3, and BubR1. The RZZ complex however is required for the recruitment of two other proteins, Mad1 and Mad2. Mad2 can be found in two different conformations within the cell, unbound, open and inactive (O-Mad2) as well as bound with Mad1, active and closed (C-Mad2) (113, 114). In this closed conformation, a heterotetramer is formed between two Mad1 and two C-Mad2

proteins, however O-Mad2 is also found floating around within the cytoplasmic. Due to Mad2's ability to dimerize, the active C-Mad2 in the Mad1-C-Mad2 complex is able to bind free O-Mad2, converting it to C-Mad2. This newly created C-Mad2 is then able to bind Cdc20 thereby creating a Cdc20-C-Mad2 complex, which is the first step in the creation of a mitotic checkpoint complex (MCC) (115) . As Cdc20 is required for APC/C activity, this sequestration functions as the predominant APC/C inhibitor and thus the arrest of mitosis. This process is possible due to the activity of two kinases. In the early stages of mitosis, the kinase, Mps1, promotes RZZ recruitment to the kinetochore, which in turn recruits Mad1-C-Mad2 (116). Later, it will release itself and recruit free O-Mad2. If a spindle is properly attached to a kinetochore, Mad1-C-Mad2 is inhibited by the SAC inhibitor, $p31^{comet}$, binding C-Mad2 in its region responsible for dimerization. This inhibition is referred to as capping the complex because it blocks the ability to recruit O-Mad2 and thus the formation of the Cdc20-C-Mad2 complex (117). It is thought that Mps1 might phosphorylate $p31^{comet}$ thereby releasing the inhibition of the complex and allow recruitment of O-Mad2 (118). Additionally, if the spindle is unattached to the kinetochore, phosphorylation of Mad1 by Bub1 results in its recruitment to the RZZ. Following the formation of the Cdc20-C-Mad2 complex, C-Mad2 will directly interact with the KEN box of BubR1 of the BubR1-Bub3 complex using the same region it would use to dimerize with O-Mad2 (119). Further, TPR domains in BubR1 also directly interact with Cdc20. Finally, this complex consisting of C-Mad2, Cdc20, BubR1, and Bub3 forms a complete MCC and engages the APC/C. The MCC is thought to interfere with APC/C's substrate binding in one of two ways; MCC induces a conformational change in APC/C or it directly occupies the substrate binding cite. This interference blocks APC/C's ability to recruit and bind cyclin B1 and securin thereby

halting the transition to anaphase. Upon successful and stable microtubule attachment to the kinetochore, the MCC is removed and thus SAC inactivated by a process called stripping. This stripping is accomplished by dynein and results in the removal of the RZZ, Mad1, and Mad2 from kinetochores (120). A now free APC/C can polyubiquinitate cyclin B, thereby leading to MPF disassembly and the progression towards the end of mitosis. APC/C can additionally target securin for degradation allowing separase to cleave cohesin and the separation of sister chromatids. As MPF activity decreases, the sites within myosin that were phosphorylated become dephosphorylated allowing cytokinesis to occur.

Concluding Thoughts

This chapter has attempted to provide an overview of how both cellular apoptosis and proliferation are regulated in the hopes that understanding both mechanisms can instill insight into how neurons might die through aberrant cell cycle re-entry. Unfortunately, however, these processes are more complex than what has been written and not all detail could be included. Once such omission is the introduction of a highly abundant and ubiquitous nucleolar phosphoprotein known as Nucleophosmin 1. This protein is critical for cell proliferation and regulates many other cellular processes including ribosome biogenesis and the DNA-damage response. While it is a heavily researched molecule, much is known about its role in proliferative cells but little has been discovered about what it does in post-mitotic neurons. As such, the following chapter will provide a detailed overview of its functions within the cell and what insight it may provide into its roles in neurons.

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CHAPTER 2

INSIGHTS INTO THE REGULATION OF NEURONAL VIABILITY BY NUCLEOPHOSMIN/B23

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II. 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.

III. 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 overexpressed 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 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. 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.

IV. THE NUCLEOPHOSMIN/NUCELOPLASMIN 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).

V. THE ROLES OF NPM IN PROLIFERATIVE CELLS

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 (summarized in Table 2.1). 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 production of ribosomes, 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) (Table 2.2). Thr199 phosphorylation by CDK2 further regulates an interaction between NPM and Rho-associated protein kinase II (ROCK II).

Table 2.1. The roles of NPM in ribosome biogenesis in proliferative cells.

Interaction	Effect on / Function of NPM	Effect on the Cell	Reference
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 Inhibition of 28S maturation	23, 24
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 CRM1 complex	Transport and export of ribosomal subunits to cytoplasm	Ribosome production	3, 16, 17
CK2	Phosphorylation by CK2 promotes dissociation of substrate bound to NPM	Inhibition of NPM chaperone activity	15

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) (Table 2.2). 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).

Table 2.2. The roles of NPM in centrosome duplication in proliferative cells.

Interaction	Effect on / Function of NPM	Effect on the Cell	Reference
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

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 (summarized in table 2.3). 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 (Table 2.4).

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

Table 2.3. Regulation of the ARF/MDM2/p53/p21 pathway by NPM in proliferative cells

Interaction	Effect on / Function of NPM	Effect on the Cell	Reference
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

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).

Table 2.4. The roles of NPM in oxidative stress and DNA damage in proliferative cells

Interaction	Effect on / Function of NPM	Effect on the Cell	Reference
pRb and E2F1	Sumoylated NPM interacts with hyperphosphorylated pRb in nucleoli during late S-phase/early G_2 Upon uv-induced damage, phosphorylated NPM associates with pRb, blocking repression of E2F1	Increased E2F1 transcriptional activity	23, 54, 55
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

VI. THE ROLE OF NPM IN THE REGULATION OF APOPTOSIS

Signaling pathways

NPM has been connected to the pro-survival nature of the PI3-K/Akt and MAPK/ERK signaling pathways (summarized in Table 2.5). 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

Table 2.5. The regulation of apoptosis of proliferative cells by NPM.

Interaction	Effect on / Function of NPM	Effect on the Cell	Reference
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	

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 (summarized in Table 2.5). 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 withdrawl 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.[1]

[1] Unpublished data

VII. 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 anaemia 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 above, 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 as well as $NPM^{-/-}$ 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).

VIII. THE ROLE OF NPM IN NEURONS

The information described until now has mostly concerned NPM's role in proliferative cells. 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 Tables 2.6 and 2.7). 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

Table 2.6. The role of NPM in models of neuronal death and injury

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 treamtment	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 NMDA treated primary cortical neurons	Protection against SIAH1-GAPDH induced death	NPM disrupts SIAH1-GAPDH complex. Overexpression in cortical neurons and mice inhibited NMDA induced toxicity by inhibiting SIAH1	85
Kainic acid (KA)-induced excitiotoxicity in rats	KA treated primary cortical neurons and SH-SY5Y cells NE-4C cells	Decreased NPM shows increased p53 levels in KA-treated SH-SY5Y cells Apoptosis following NPM downregulation is p53-independent	Downregulation of NPM protein in degenerating neurons of the hippocampal CA1 region of KA-treated rats and KA-treated primary cortical neurons	86

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.[2] 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,

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[2] Unpublished data

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) (Table 2.4).

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 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.

Table 2.7. The roles of NPM in neuronal nucleolar stess

In vivo model	*In vitro* model	Impact on cell viability	Effect on NPM	Reference
	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 p53 prolongs survival through PTEN upgregulation, inhibition of mTOR signaling and activation of autophagy	Nucleoplasmic relocalization from nucleoli	114

IX. 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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CHAPTER 3

REGULATION OF NEURONAL SURVIVAL BY NUCLEOPHOSMIN 1 (NPM1) IS DEPENDENT ON ITS EXPRESSION LEVEL, SUBCELLULAR LOCALIZATION AND ITS OLIGOMERIZATION STATUS

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II. ABSTRACT

Nucleophosmin 1 (NPM1) is a nucleolar phosphoprotein that regulates many cellular processes, including ribosome biogenesis, proliferation and genomic integrity. Although its role in proliferating cell types and tissues has been extensively investigated, little is known about its function in neurons and in the brain where it is highly expressed. We report that NPM1 protein expression is increased selectively in the striatum in both the R6/2 transgenic and 3-nitropropionic acid (3-NP)-injected mouse models of Huntington's disease (HD). Examination of the effect of ectopic expression on cultured neurons revealed that increasing NPM1 is toxic to otherwise healthy cerebellar granule and cortical neurons. Toxicity is dependent on its cytoplasmic localization and oligomerization status. Forced retention of NPM1 in the nucleus, as well as inhibiting its ability to oligomerize, not only neutralizes NPM1 toxicity, but renders it protective against apoptosis. While not blocked by pharmacological inhibition of the proapoptotic molecules, JNK, GSK3β or caspases, toxicity is blocked by compounds targeting cyclin dependent kinases (CDKs) as well as by dominant negative forms of CDK1 and CDK2 and the pan-CDK inhibitor, $p21^{Cip1/Waf1}$. While induced in *in vivo* HD models, NPM1's protein levels are unchanged in cultured cerebellar granule and cortical neurons induced to die by low potassium or homocysteic acid treatment, respectively. Moreover, and counterintuitively, knockdown of its expression or inhibition of endogenous NPM1 oligomerization in these cultured neurons is toxic. Taken together, our study suggests that while neurons need NPM1 for

survival, an increase in its expression beyond physiological levels and ability to translocate is harmful and leads to cell cycle induction.

III. INTRODUCTION

NPM1, also known as B23, is a 32 kDa protein that is an abundant, ubiquitously expressed, and evolutionarily conserved non-ribosomal nucleolar phosphoprotein (1, 2). It is one of three members of the nucleophosmin/nucleoplasmin family of proteins, the other two being NPM2 and NPM3. NPM1 is an important regulator of a variety of cellular processes including centrosome duplication, genomic stability, cell proliferation and the response to genotoxic stress.

Inactivation of the NPM1 gene in mice causes death at mid-gestation indicating its requirement for normal development. Fibroblasts cultured from NPM1 knockout mice display genomic instability and reduced growth and cell proliferation (3). Other studies have shown that NPM1 can shuttle between the nucleolus to the nucleoplasm and from the nucleus to the cytoplasm. Nuclear export is believed to be required for cellular proliferation (4, 5). Mutations of NPM1 are associated with cancers and are responsible for about a third of cases of acute myeloid leukemia (AML) in humans (3, 6–8). These mutations, which result in an altered C-terminus containing an addition of a nuclear export signal and termed NPMc+ have been reported to aberrantly localize NPM1 to the cytoplasm (8). Overexpression of NPM1 which results in elevated cytoplasmic localization, has been shown to promote cell transformation (9, 10).

Much of the research on NPM1 has been performed using proliferating cells and tissue types, particularly in the context of cancer (1–3). However, it is highly expressed in the brain and in neurons where its function is poorly understood. Emerging evidence indicates that the nucleolus, of which NPM1 is a prominent resident, does play an important role in neuronal development

and maintenance, and nucleolar dysfunction has been implicated in various neurodegenerative diseases (11, 12). This suggests that NPM1 may influence the regulation of neuronal survival. Indeed, two separate studies have described anti-apoptotic effects of NPM1 when overexpressed in cell lines of neuronal origin (13, 14). However, the contribution of NPM1 to the regulation of survival in primary neurons is less clear. We have examined this issue using two types of CNS neurons, cerebellar granule and embryonic cortical neurons. Our results suggest that NPM1 plays complex roles in the regulation of neuronal survival that are dependent on its level of expression, subcellular localization and the extent to which it oligomerizes.

IV. RESULTS

NPM1 is upregulated in the striatum in mouse models of HD−Since nucleolar dysfunction is a feature of neurodegenerative diseases (15–17) and given that NPM1 is a key component of the nucleolus, we investigated the status of NPM1 expression in mouse models of HD. We first examined R6/2 transgenic mice, which are the most commonly used mouse model of HD (18, 19). These mice express an N-terminus fragment of the huntingtin gene (HTT) with ~120 CAG repeats. Locomotor deficits can be observed as early as 7 weeks of age, with severe striatal atrophy and motor impairment by 12 weeks followed by death at around 15 weeks (18, 19). While NPM1 expression in R6/2 mice is elevated in the cortex, it is comparable in the rest of the brain to wild-type littermates at 6 weeks (Fig. 3.1). However, at 10 weeks it is elevated in both the cortex and striatum, coinciding with the onset of neuropathology (Fig. 3.1). Interestingly, in non-striatal tissue (see other brain parts, OBP), where there is limited or no neuropathology, expression is unchanged (Fig. 3.1). We also used the 3-nitropropionic acid (3-NP) model, a chemical model of HD (20, 21). Administration of 3-NP to mice causes robust and selective striatal neurodegeneration and faithful recapitulation of key features of HD. In this model, degeneration is obvious in the striatum 3 days after 3-NP administration and is severe by 5 days. NPM1 expression is significantly elevated in the striatum within 1 day after 3-NP administration and is greatest at 3 days when degeneration is robust (Fig. 3.2). Again, expression is unchanged in OBP after 3-NP administration (Fig. 3.2).

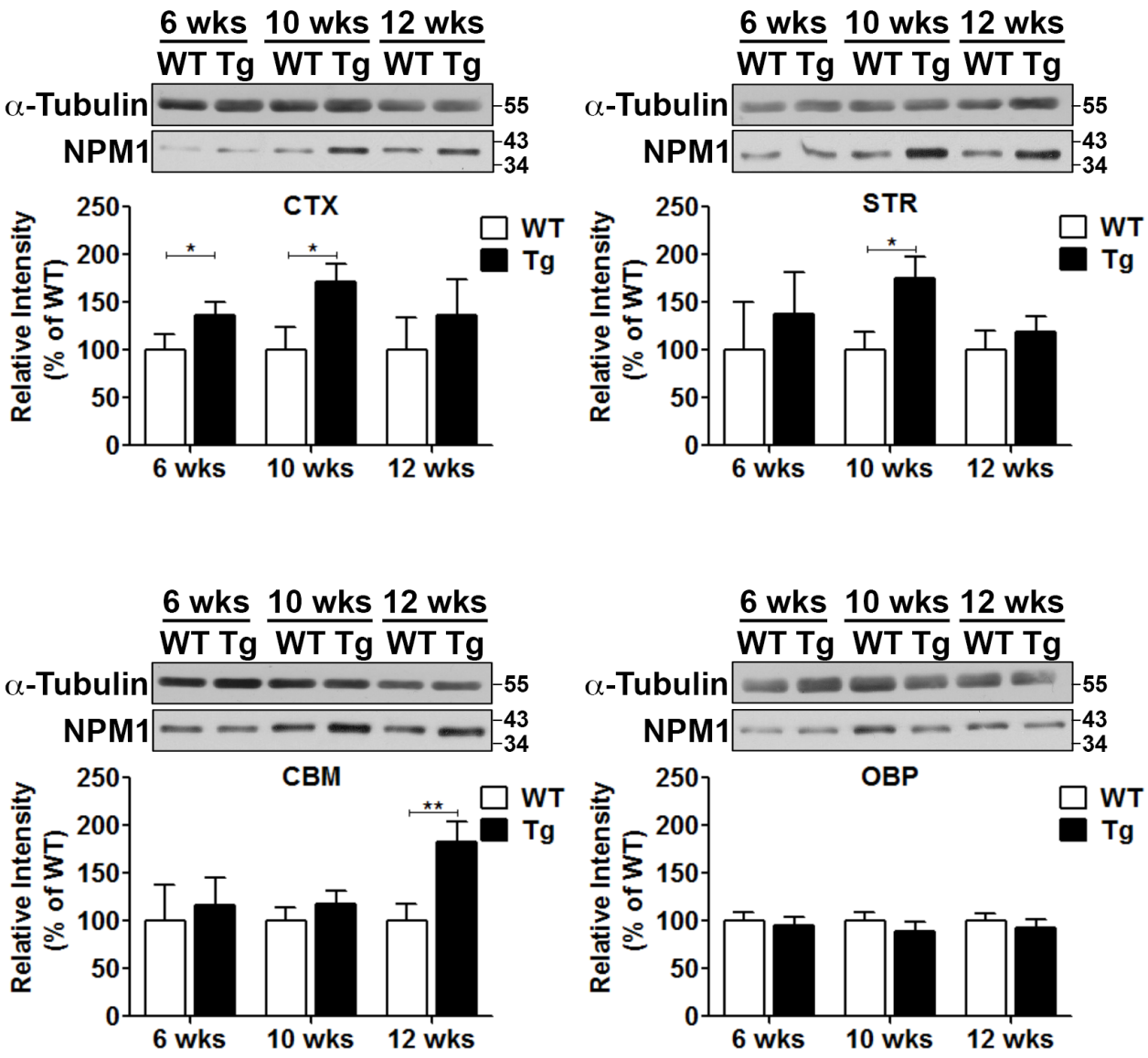


Figure 3.1. Induction of NPM1 expression in the R6/2 mouse models of Huntington's disease. NPM1 protein expression in the cortex (CTX), striatum (STR), cerebellum (CBM) and rest of the brain (OBP) from wild type (WT) and transgenic (Tg) R6/2 littermates at 6, 10 and 12 weeks of age. α-Tubulin serves as a loading control. Graphs show densitometric analysis presented as means ± S.D. (n=3).

Overexpression of NPM1 kills neurons−Since NPM1 expression is increased in mouse models of HD, we examined whether increasing its expression ectopically impacted the viability of normally healthy neurons. For this, we used CGNs, which are healthy in culture when

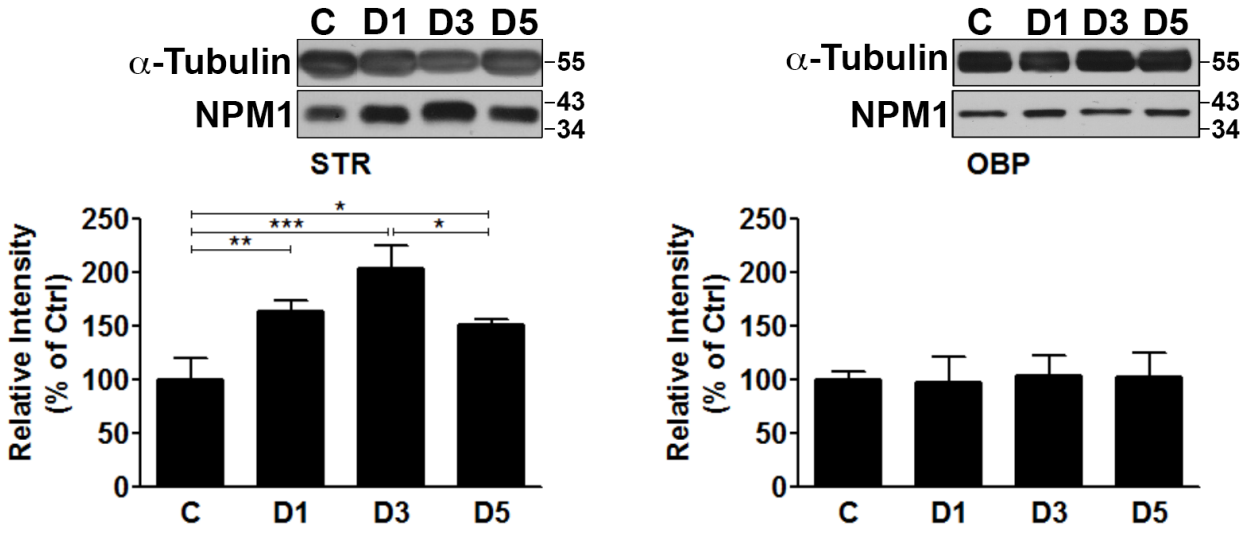


Figure 3.2. Induction of NPM1 expression in the 3-NP mouse models of Huntington's disease. Protein expression of NPM1 from the striatum (STR) and the rest of the brain (OBP) of wild type mice injected with either saline control (C) or 3-nitropropionic acid (3-NP) for 1 day (D1), 3 days (D3) or 5 days (D5). α-Tubulin serves as a loading control. Graphs show densitometric analysis presented as means ± SD (n=3).

maintained in depolarizing medium (high potassium or HK) but die when switched to non-depolarizing medium (low potassium or LK). Elevated NPM1 expression in CGNs causes death even in HK while not influencing cells already undergoing apoptosis (Fig. 3.3A). Induction of apoptosis by NPM1 was confirmed through cleaved caspase-3 co-staining (Fig. 3.3B). To confirm that this result was not specific to CGNs, we utilized a distinct neuronal type of embryonic cortical neurons, which can be induced to die through oxidative stress by treatment with homocysteic acid (HCA). Similarly, forced expression of NPM1 resulted in the death of otherwise healthy cortical neurons and did not exacerbate death due to HCA (Fig. 3.3C). In both types of neurons, ectopic NPM1 localized predominantly in the nucleus and within nucleoli when the neurons were healthy (Fig. 3.4A). However, in neurons that were dying NPM1 was localized to the cytoplasm (Fig. 3.4B), including within neurites (Fig. 3.4B). Quantitation of

NPM1 localization in relation to neuronal viability confirmed this localization pattern in both CGNs (Fig. 3.4C) and cortical neurons (Fig. 3.4D).

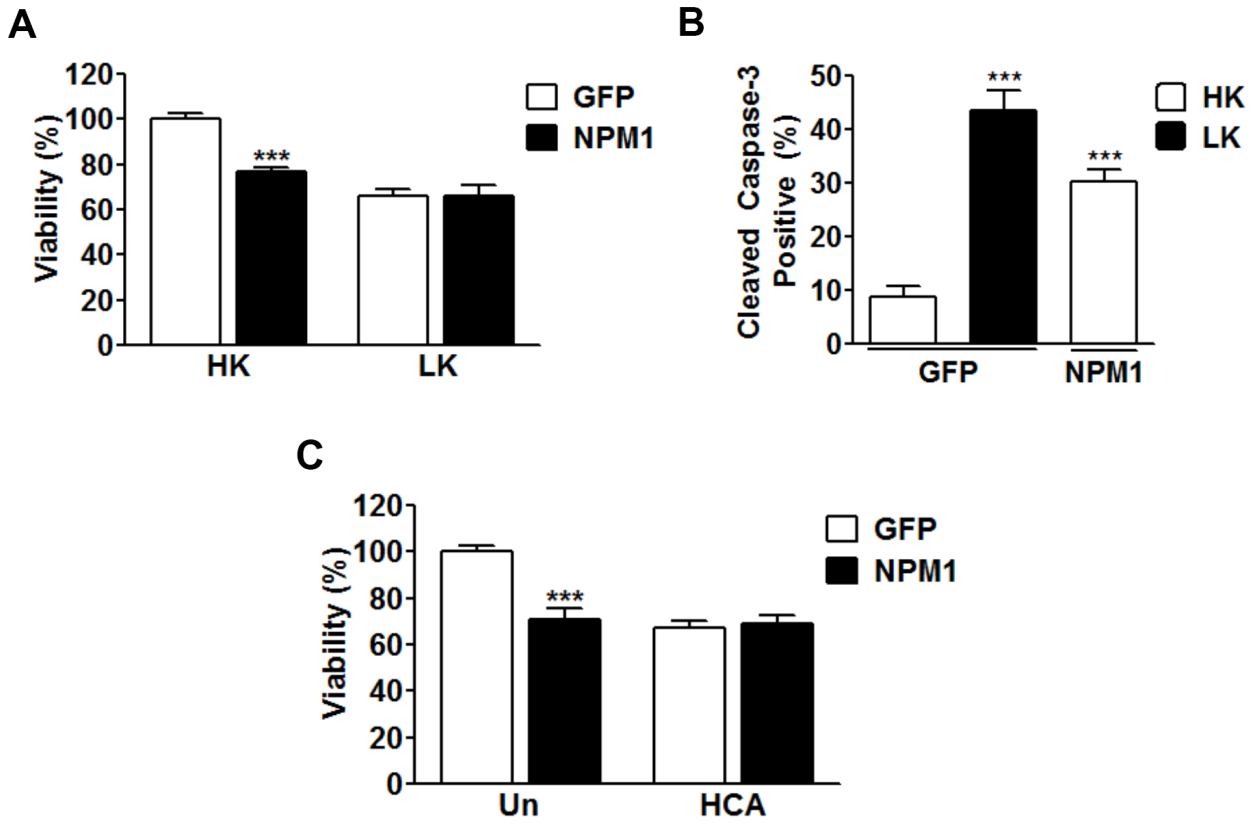


Figure 3.3. Increased expression of NPM1 is toxic to otherwise healthy neurons. *A*. CGNs transfected with either EGFP or NPM1 and treated with HK or LK media as described in experimental procedures. Viability of transfected cells was quantified by immunocytochemistry with GFP or Flag antibodies. ***, $p<0.001$ as compared to EGFP HK (n=4); *B*. CGNs transfected as performed in (A). Viability was quantified by immunocytochemistry with GFP or Flag antibodies co-stained for cleaved caspase-3. ***, $p<0.001$ as compared to EGFP HK (n=3). *C*. Cortical neurons transfected with either EGFP or NPM1 for 8 h and then either left untreated (Un) or treated with HCA for 15-16 h. Viability was quantified as done in (A). ***, $p<0.001$ as compared to EGFP Un (n=3).

NPM1 is neurotoxic when localized to the cytoplasm−NPM1 is known to shuttle between the nucleolus, nucleoplasm and cytoplasm at least in proliferating cell types (4, 5). As described above, in dying neurons NPM1 localized preferentially to the cytoplasm. To investigate the

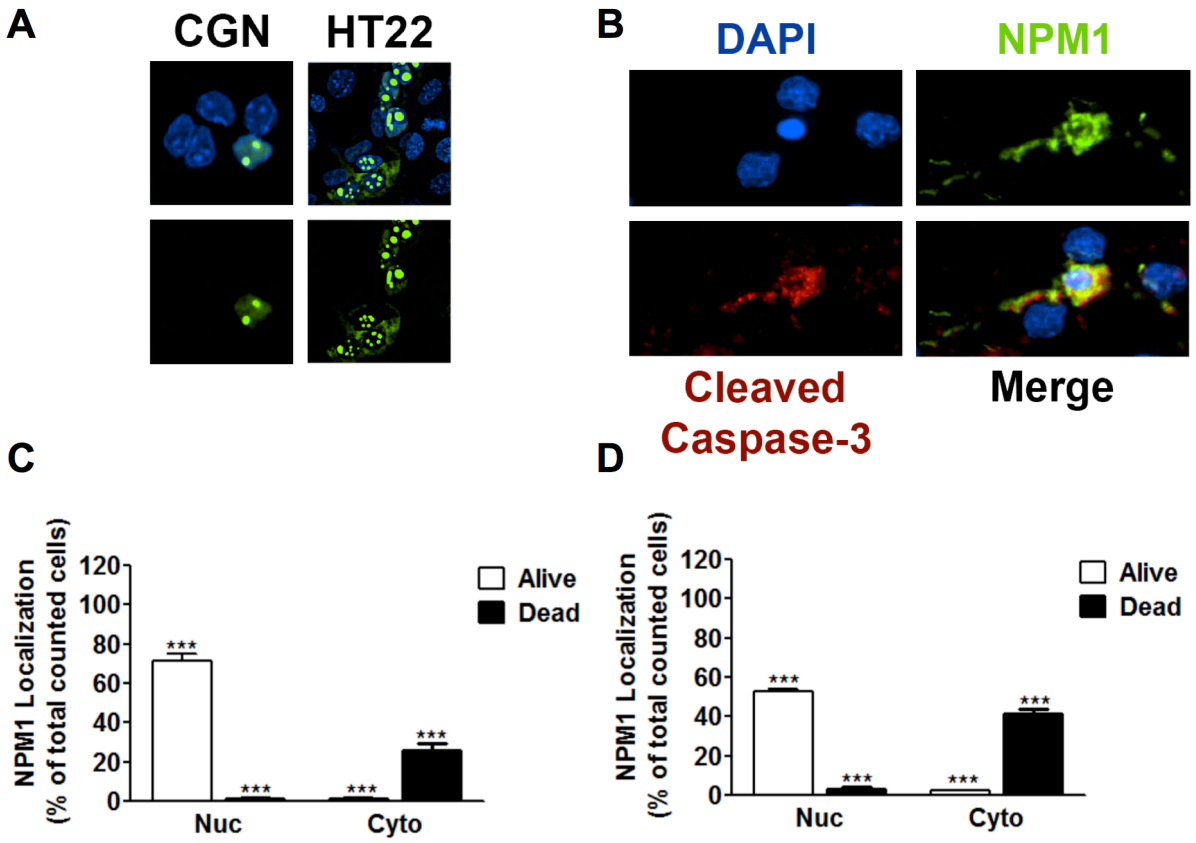


Figure 3.4. NPM1 localizes to the cytoplasm of dead neurons. *A*. Localization of ectopic NPM1 expression in living cells. NPM1 was transfected into CGNs (left) or HT22 cells (right) for 24 h. CGNs were then treated with HK media for an additional 24 h. Cells were then fixed, DAPI stained and NPM1 was imaged by EGFP autofluorescence. *B*. Representative image showing localization of ectopic NPM1 in apoptotic neurons from (Fig. 3-3 B). NPM1 displayed a nucleolar and nucleoplasmic localization in healthy living cells and a cytoplasmic localization in dead cells. *C & D*. Quantitation of NPM1 localization in relation to cell viability from CGNs transfected in (Fig. 3-3 B) and cortical neurons transfected in (Fig. 3-3 C). Each graph represents ≥ 800 cells counted. ***, $p<0.001$ (n=3).

contribution of intracellular localization to neurotoxicity, we utilized four mutant NPM1 constructs, denoted as NLSD, NESD, NESM and NoLSM, whose localization was restricted to either the cytoplasm or nucleus (22). NLSD contains a 17 amino acid deletion (residues 141-157) of NPM1's bipartite nuclear localization signal (NLS), whereas NESD has a 9 amino acid deletion (residues 94-102) and NESM has L100A and L102A mutations of the nuclear export

signal (NES) (Fig. 3.5). NoLSM contains W288G and W290G mutations in NPM1's nucleolar localization signal (Fig. 3.5). As described previously by Wang et al. (2005) (22), the localization of NLSD is cytoplasmic and nucleolar but absent from the nucleoplasm, whereas NESD, NESM and NoLSM are nuclear (Fig. 3.6A). When expressed in CGNs, NLSD displayed toxicity similar to wild-type NPM1 under both HK and LK conditions (Fig. 3.6B). In contrast, restricting NPM1 to the nucleus using NESD or NESM reduced the toxicity of NPM1 in HK substantially (Fig. 3.6B). Indeed, both NESD and NESM showed a significant level of protection in LK-treated neurons. Mutation of its nucleolar localization signal rendered NPM1 less toxic in HK, however, and offered modest protection under LK conditions (Fig. 3.6B). Similarly, these effects of the mutants on neuronal viability were also seen in cortical neurons treated with HCA (Fig. 3.6C). Along with the cytoplasmic localization of NPM1 in dying neurons, these results suggest that neurotoxicity by NPM1 requires its cytoplasmic location.

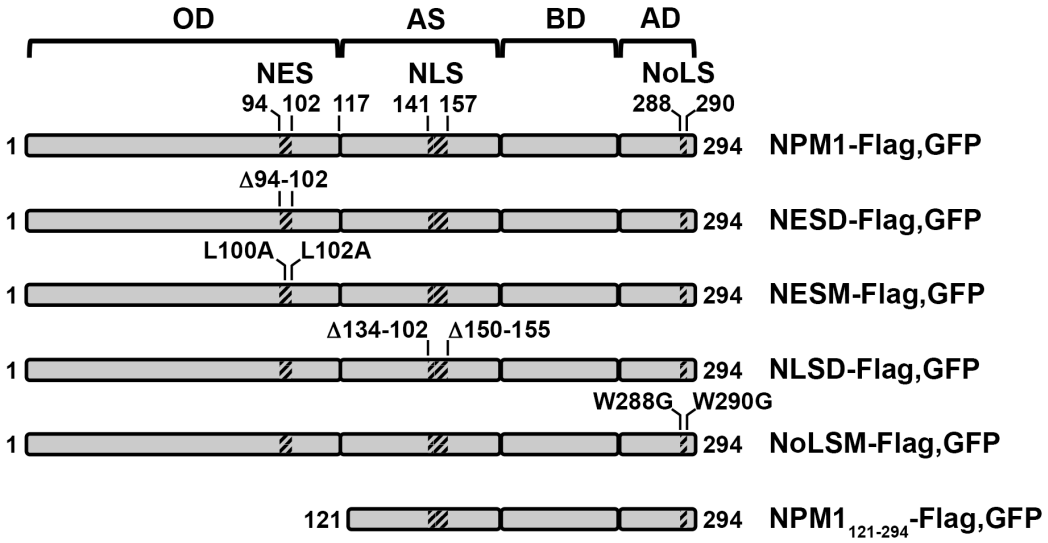


Figure 3.5. Schematic detailing the constructs used in this study (adapted from Wang et al. 2005). OD: N-terminal oligomerization domain, AS: acidic stretches, BD: basic domain, AD: aromatic domain.

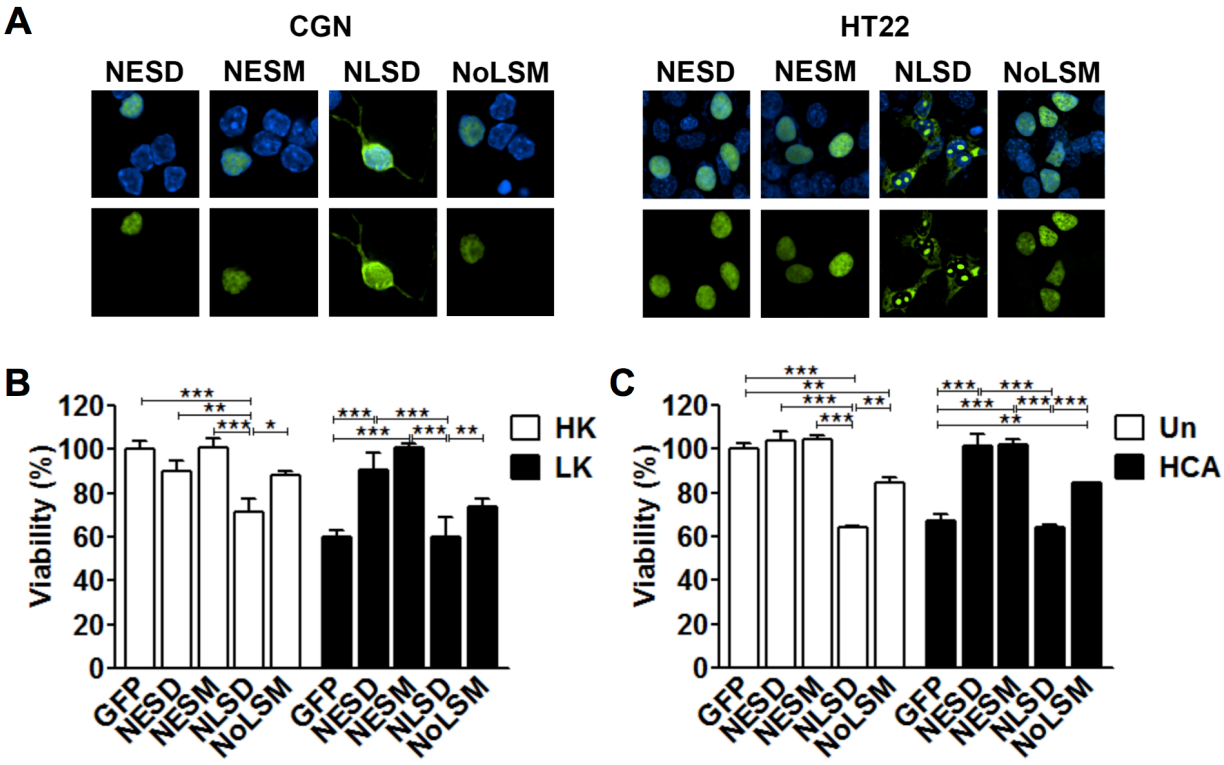


Figure 3.6. Effect of NPM1 on neuronal viability is dependent on its cellular localization. *A*. Localization of NPM1 mutants in CGNs (left panel) and HT22s (right panel). NESD, NESM, NLSD or NoLSM mutants were transfected into CGNs for 24 h, followed by 24 h HK treatment, or HT22s for 24 h. Cells were then fixed, DAPI stained and NPM1 localization was imaged by EGFP autofluorescence. *B*. CGNs transfected with EGFP, NESD, NESM, NLSD or NoLSM and treated with HK/LK media. Viability was quantified by immunocytochemistry with GFP or Flag antibodies and DAPI staining. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (n=3). *C*. Cortical neurons transfected with EGFP and the four NPM1 mutants for 8 h followed by HCA treatment for 15-16 h. Viability was quantified as just described. **, $p<0.01$; ***, $p<0.001$ (n=3).

Oligomerization of NPM1 promotes neuronal death−NPM1 forms pentameric oligomers through an oligomerization domain mapped to residues 15 – 118 within the protein (23, 24). Aptamers have been identified that inhibit NPM1 oligomerization both *in vitro* and in cells (25). Expression of these aptamers in cancer cells not only increases their sensitivity to DNA-damaging agents but is sufficient to promote apoptosis of these cells (25). To investigate the significance of NPM1 oligomerization to its regulation of neuronal viability, we utilized two

aptamers previously shown to block NPM1 oligomerization by Jian et al. (25). We independently confirmed that these aptamers, 1A1 and $1A1_{1\text{-}40}$ (designated here as $1A1_{Trunc}$), both inhibited NPM1 oligomerization (Fig. 3.7A). While neither aptamer had an influence on the viability of CGNs undergoing apoptosis, both had a slight toxic effect on the viability of healthy neurons, which was only significant with $1A1_{Trunc}$ (Fig. 3.7B). However, when co-

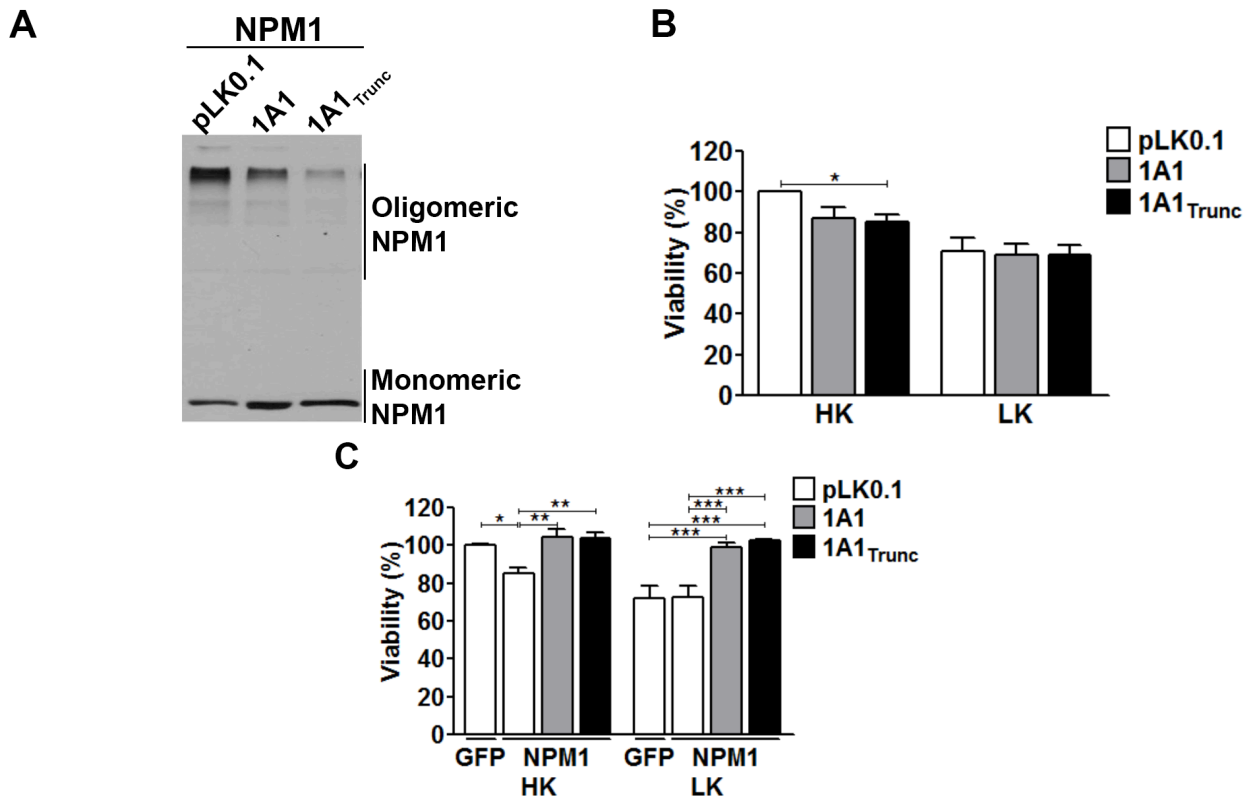


Figure 3.7. Blocking NPM1 oligomerization is protective. *A*. NPM1 RNA aptamers inhibit NPM1 oligomerization. NPM1 was co-transfected into HEK293T cells with either a control plasmid (pLK0.1), 1A1 or $1A1_{Trunc}$ in a 1:2 ratio for 24 h. Cells were then lysed and subjected to crosslinking as described in experimental procedures. *B*. CGNs were transfected with either pLK0.1 co-expressing with EGFP in a 6.5:1 ratio, 1A1 or $1A1_{Trunc}$ for 48 h followed by HK/LK treatment. Viability was quantified based on either EGFP (pLK0.1) or DsRed (aptamers) autofluorescence. *, $p<0.05$ (n=3). *C*. CGNs transfected with EGFP and pLK0.1 or NPM1 and pLK0.1, 1A1 or $1A1_{Trunc}$ in a 1:2 ratio for 48 h followed by HK/LK. Viability was quantified by immunocytochemistry based on EGFP or NPM1 transfection with GFP or Flag antibodies. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (n=3).

expressed with NPM1, not only was NPM1 no longer toxic in HK, it fully protected CGNs from LK-induced cell death (Fig. 3.7C) suggesting that while elevated expression by itself was not toxic, the oligomerization of NPM1 transformed it from a protective molecule to a toxic one. To further investigate this issue, we expressed an NPM1 incapable of oligomerizing due to a deletion of its N-terminal oligomerization domain ($NPM1_{121-294}$) (Fig. 3.5). Consistent with oligomerization being needed for toxicity rather than elevated levels, overexpression of monomeric NPM1 no longer induced death in healthy CGNs (Fig. 3.8A) and cortical neurons (Fig. 3.8B). As was seen by co-expression with the aptamers, $NPM1_{121-294}$ was fully protective under apoptotic conditions (Fig. 3.8A and B). We tested the ability of NESD, NESM, NLSD, NoLSM and $NPM1_{121-294}$ to oligomerize. As expected, $NPM1_{121-294}$ failed to oligomerize (Fig. 3.9). In comparison with wild-type NPM1, both NoLSM and NLSD displayed a similar ability to oligomerize (Fig. 3.9). In contrast, NESD and NESM showed no oligomerization (Fig. 3.9). Taken together, these results suggest that oligomerization of NPM1 occurs in the cytoplasm and that it is necessary for the neurotoxicity of NPM1.

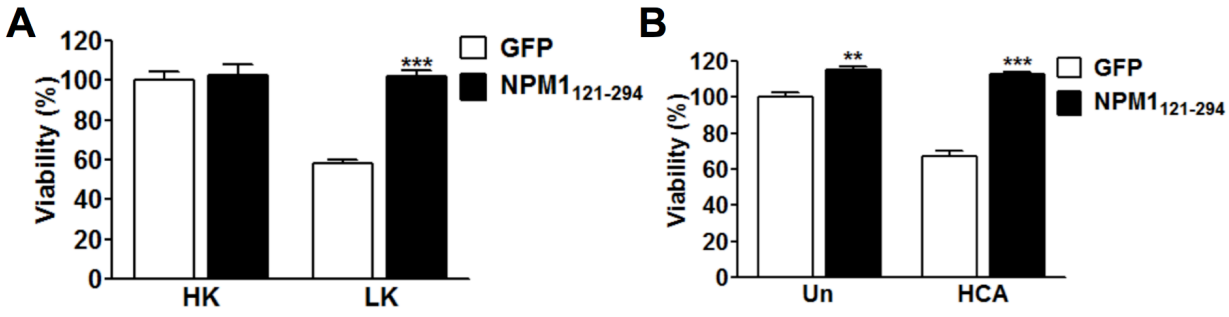


Figure 3.8. Monomeric NPM1 is protective against apoptosis. *A and B*. EGFP or $NPM1_{121-294}$ were transfected into CGNs and treated with HK/LK (A) or cortical neurons and either left untreated (Un) or treated with HCA (B) as previously described. Viability quantification was performed as done in (Fig 3-7 C). **, $p<0.01$ untreated NPM1 transfected neurons compared to untreated EGFP transfected neurons; ***, $p<0.001$ $NPM1_{121-294}$ transfected neurons compared to EGFP transfected neurons in LK and HCA (n=3).

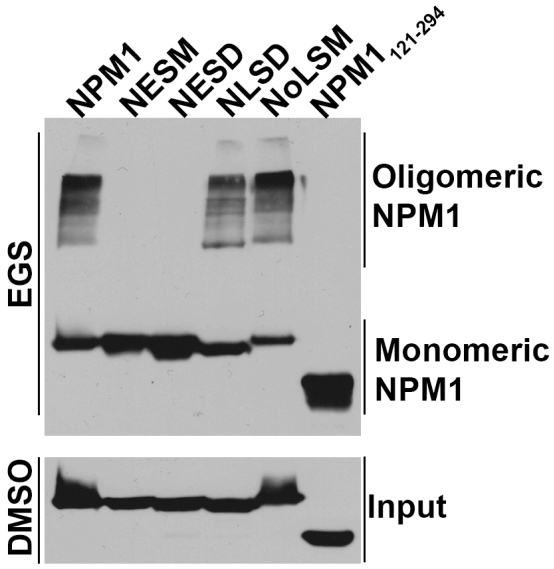


Figure 3.9. Crosslinking of NPM1 mutants transfected into HEK293T cells for 24 h as described in experimental procedures.

Overexpressed NPM1 kills by promoting abortive cell cycle re-entry−Several studies performed in cell lines have found that NPM1 promotes cell cycle progression, particularly when it is in the cytoplasm. In fact, nuclear export is necessary for cell cycle progression by NPM1 (4, 5, 10). Abortive cell cycle re-entry is a well-described mechanism of neuronal death (26–28). We therefore investigated whether NPM1-induced toxicity involved activation of the cell cycle machinery. Treatment with both roscovitine, a widely used and broad spectrum CDK inhibitor (29), as well as another CDK inhibitor, HSB13 (30), protected against NPM1 neurotoxicity (Fig. 3.10A). Similarly, co-expression of dominant-negative forms of either CDK1 or CDK2 inhibited NPM1 neurotoxicity (Fig. 3.10B). Furthermore, the toxicity from NLSD expression is blocked by both treatment with roscovitine and expression of dominant negative CDK1 and CDK2 (Fig. 3.10C and D). Activation of caspases, JNK, and GSK3β have also been shown to be important

for neuronal death (31–33). In contrast to the CDK inhibitors, however, inhibitors targeting these pro-apoptotic molecules had no protective effect (Fig. 3.10A). These results suggest that the toxic effect of elevated NPM1 in neurons is mediated by cell cycle activation.

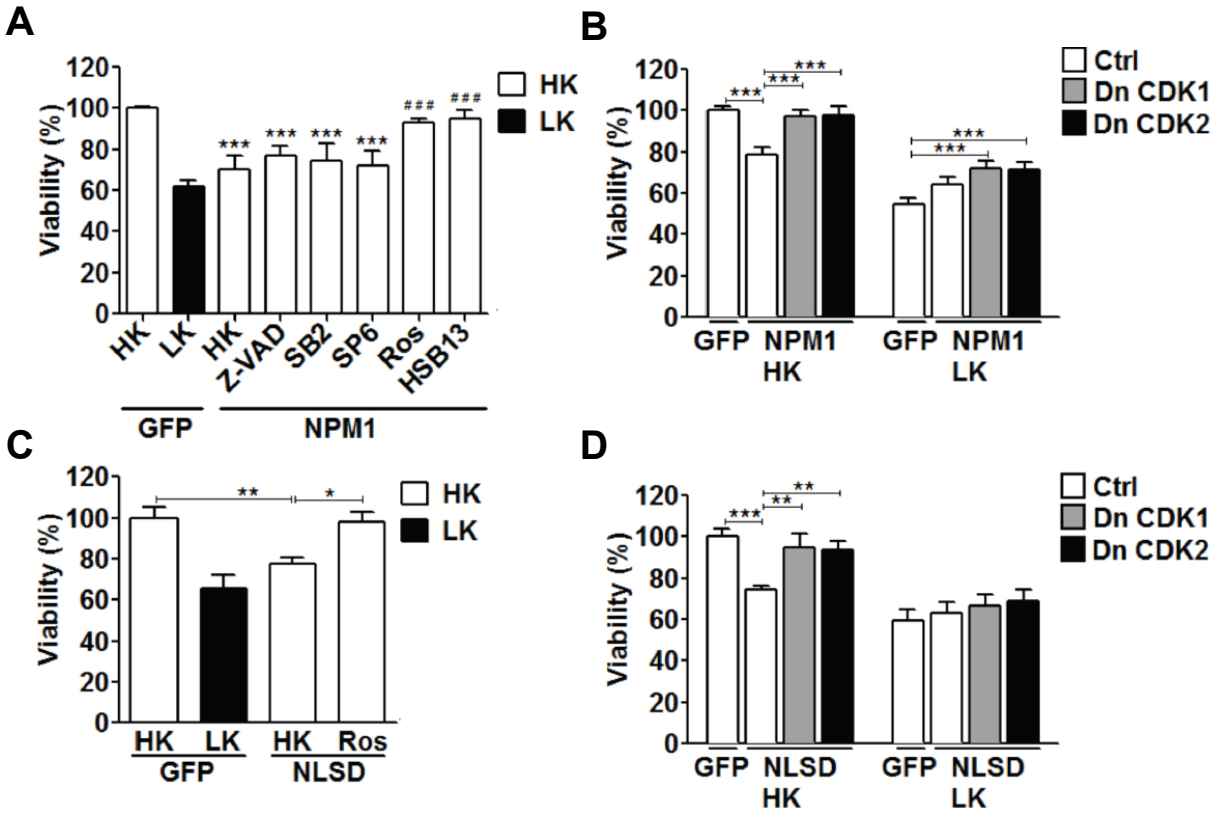


Figure 3.10. Toxicity by increased NPM1 expression is cell cycle dependent. *A*. CGNs transfected with either EGFP or NPM1. Cells were then treated with either HK media or HK media supplemented with the following inhibitors: Z-VAD (50 μM), SB216763 (5 μM), SP600125 (10 μM), Roscovitine (50 μM) or HSB13 (25 μM). Viability was quantified by immunocytochemistry with a GFP or Flag antibody. ***, $p<0.001$ as compared to GFP in HK; ###, $p<0.001$ as compared to NPM1 treated with HK (n=3). *B*. CGNs were transfected with either EGFP and a control vector (Ctrl, pK3HA) or NPM1 and pK3HA (Ctrl), DnCDK1, or DnCDK2 (B) in a 1:2 ratio followed by HK/LK treatment. Viability was quantified based on NPM1 fluorescence by immunocytochemistry with a GFP antibody. ***, $p<0.001$ (n=3). *C*. CGNs transfected with EGFP or NLSD. Cells were then treated with either HK media or HK media supplemented with Roscovitine (50 μM). Viability was quantified as described in (A). *, $p<0.05$; **, $p<0.01$ (n=3). *D*. CGNs transfected with EGFP and pK3HA (Ctrl) or NLSD and pK3HA (Ctrl), DnCDK1 or DnCDK2 in a 1:2 ratio followed by HK/LK treatment. Viability was quantified as described in (B). **, $p<0.01$; ***, $p<0.001$ (n=3).

An endogenous and physiological inhibitor of CDKs is $p21^{Cip1/Waf1}$. Indeed, $p21^{Cip1/Waf1}$ has been shown by a number of laboratories to have strong neuroprotective effects both *in vitro* and *in vivo* (34–36). Consistent with its well-established protective activity, co-expression of $p21^{Cip1/Waf1}$ blocked NPM1-induced neuronal death (Fig. 3.11).

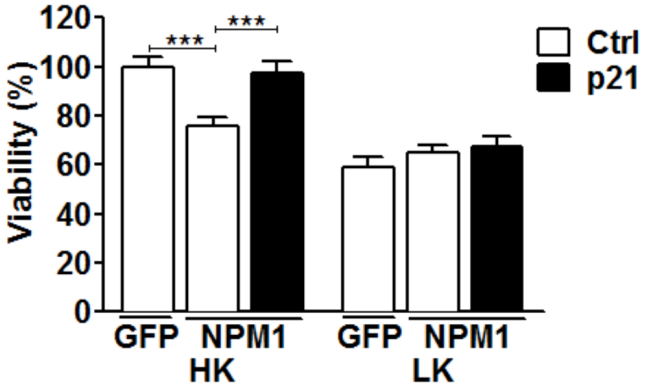


Figure 3.11. NPM1-induced death is rescued by $p21^{Cip1/Waf1}$ expression. CGNs were transfected with either EGFP and a control vector (Ctrl, pK3HA) or NPM1 and pK3HA (Ctrl), p21 in a 1:2 ratio followed by HK/LK treatment. Viability was quantified based on NPM1 fluorescence by immunocytochemistry with a GFP antibody. ***, $p<0.001$ (n=3).

Expression and significance of endogenous NPM1 to neuronal viability−As described above, NPM1 expression is elevated in mouse models of HD. Ectopically increasing its levels in cultured neurons has a neurotoxic effect. To examine if the expression of endogenous NPM1 is increased in cultured neurons induced to die, we looked at its protein levels in LK-treated neurons. As shown in Fig. 3.12A, there is no significant change in expression of NPM1 protein. Similarly, in cortical neurons induced to die by HCA treatment, NPM1 expression remains unaltered (Fig. 3.12B). Immunocytochemical analysis of expression showed no discernible changes in the number on NPM1-positive nucleoli or the intensity of staining of the nucleoli in neurons that appeared to be relatively healthy (data not shown). Interestingly and consistent with

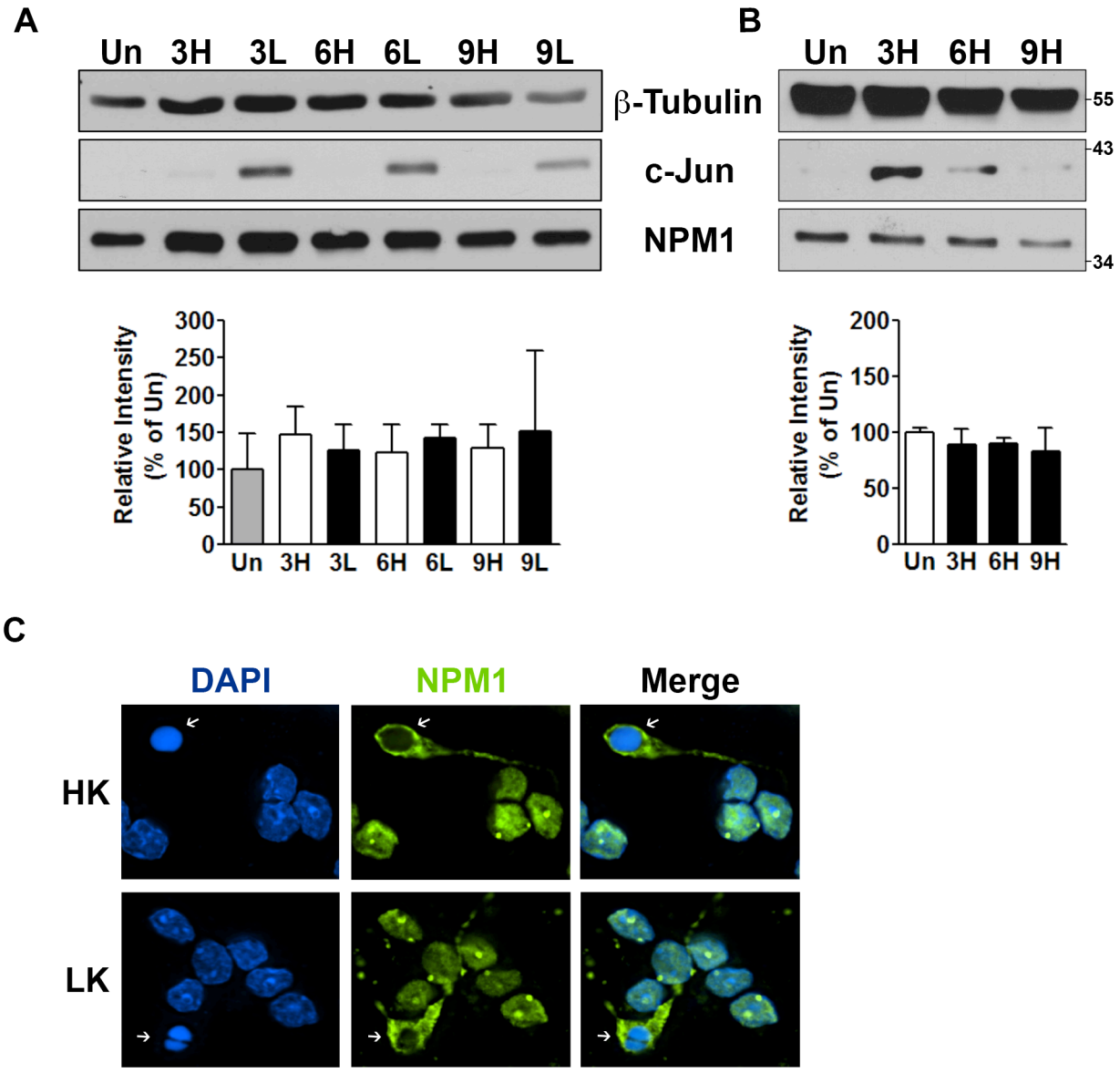


Figure 3.12. Expression pattern of endogenous NPM1. A *and B*. Protein expression (top) and densitometric analysis (bottom) presented as means ± S.D. of endogenous NPM1 in CGN (A, n=5) and Cortical (B, n=3) cultures. Cells were either left untreated (Un) or treated with HK/LK media (CGNs) or HCA (Corticals) for 3, 6 and 9 h. Endogenous NPM1 expression was analyzed as described in experimental procedures. β-Tubulin serves as a loading control and c-Jun serves as a marker for induction of apoptosis. *C*. Representative picture showing localization of endogenous NPM1 in 6 h HK/LK treated CGNs. Arrows indicate NPM1 localized to the cytoplasm of apoptotic cells.

the idea that NPM1 localization to the cytoplasm induces neurotoxicity, NPM1 was cytoplasmic in dying neurons (Fig. 3.12C).

We proceeded to determine if knocking down NPM1 influenced death of cultured neurons. We first tested the effectiveness of three different shRNAs to suppress NPM1 expression in HT22 cells, a mouse neuroblastoma cell line that is easily transfected. Two of the shRNA constructs tested, sh1 and sh2, were effective in knocking down NPM1 (Fig. 3.13A). When transfected into CGNs, both sh1 and sh2 induced death in otherwise healthy neurons (Fig. 3.13B). Knockdown of NPM1 with sh1 and sh2 also reduced survival of cortical neurons (data not shown). This suggested that while high levels of NPM1 is detrimental to neuronal survival, NPM1 at moderate levels is necessary for the viability of neurons.

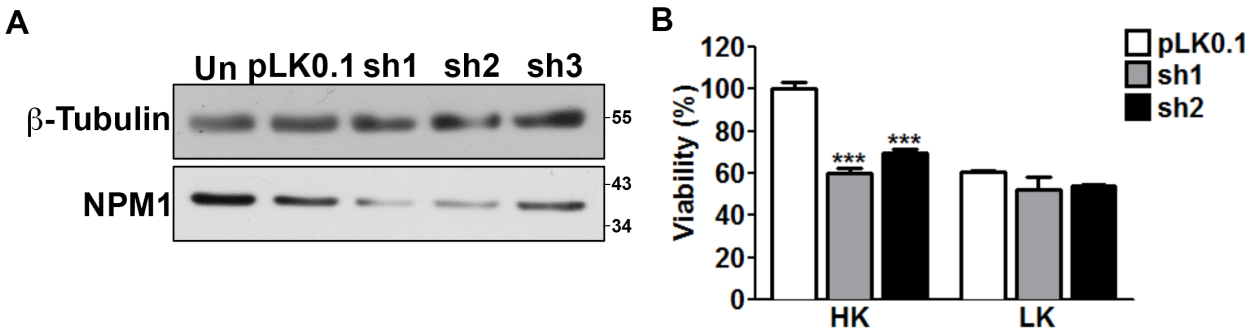


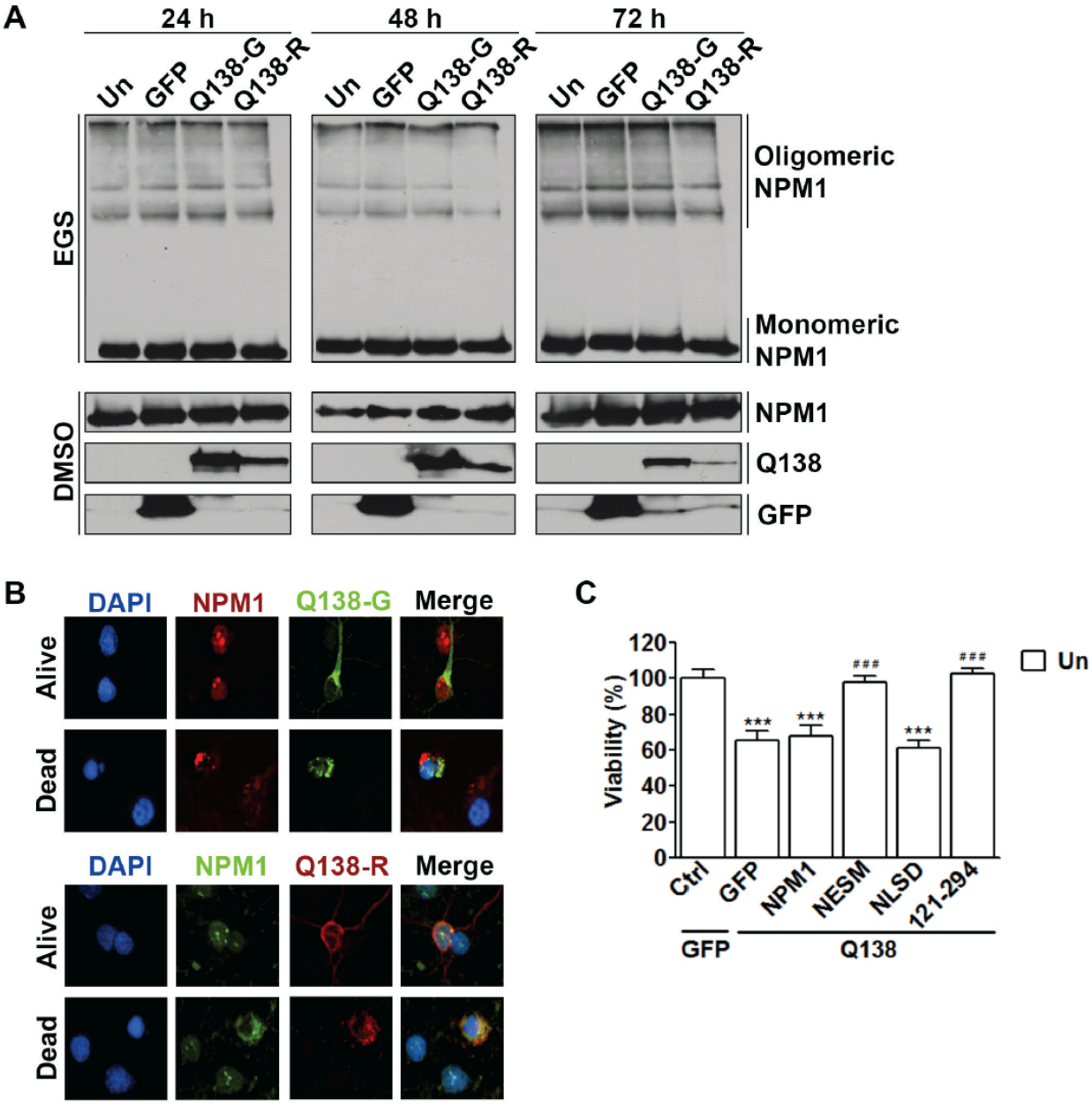
Figure 3.13. Knockdown of endogenous NPM1 is toxic to otherwise healthy neurons. *A*. HT22 cells were transfected with either a control (pLK0.1) or three commercially available shRNAs targeting NPM1 (sh1, sh2 and sh3) for 72 h. Cells were then lysed and subjected to western blotting analysis with an antibody against NPM1. β-Tubulin serves as a loading control. *B*. Viability of CGN cultures transfected with a control (pLK0.1), sh1 or sh2 along with EGFP in a 6.5:1 ratio and treated with HK/LK media. Viability was quantified by EGFP autofluorescence. ***, $p<0.001$ as compared to pLK0.1 in HK (n=3).

Nuclear NPM1 is protective against mHTT-induced cell death–As described above, NPM1 expression is increased in the cortex and striatum of symptomatic R6/2 transgenic mice (Fig. 3.1). We therefore examined whether mutant huntingtin (mHTT) itself had an effect on

endogenous NPM1 oligomerization and localization. The mouse neuroblastoma Neuro2a (N2a) cell line is sensitive to mHTT (37). Expression of two mHTT constructs (Q138-GFP and Q138-RFP) into these cells showed no change in endogenous NPM1 oligomerization over a 72 h period (Fig. 3.14A).

We proceeded to investigate if the localization of NPM1 is important for mHTT-induced toxicity of cortical neurons. While we find endogenous NPM1 localized to the cytoplasm of dead cells (Fig. 3.12C), interestingly in neurons expressing either Q138-GFP or Q138-RFP, NPM1 showed a nuclear pattern in both living and dead cells (Fig. 3.14B). Toxicity in cortical neurons from mHTT expression occurs within 24 h. To examine the effect of NPM1 localization on this toxicity, mHTT was expressed along with the different NPM1 constructs. Toxicity by mHTT was not influenced by wild-type NPM1, but was slightly increased when co-expressed with the cytoplasmic localized NPM1 (Fig. 3.14C). However, nuclear-restricted and monomeric NPM1 (NESM and $NPM1_{121-294}$) was highly protective against mHTT toxicity (Fig. 3.14C). These results suggest that mHTT does not directly influence NPM1 but instead NPM1's localization may play a role in mHTT-induced neuronal death.

Next page: Figure 3.14. Nuclear and monomeric NPM1 protects against mHTT toxicity. *A*. N2a cells were either left untransfected (Un) or transfected with EGFP, Q138-GFP or Q138-RFP. Cells were lysed 24, 48 or 72 h later and subjected to crosslinking as described in experimental procedures. Blots were probed for endogenous NPM1 and then re-probed with GFP and RFP antibodies. *B*. Cortical neurons were transfected with either Q138-GFP (top panel) or Q138-RFP (bottom panel) and cells were fixed 24 h later. Immunocytochemistry was performed by co-staining with NPM1 and either GFP or RFP antibodies. *C*. Cortical neurons transfected in a 1:2 ratio with either EGFP and a control vector (pK3HA) or Q138-RFP and EGFP, NPM1, NESM, NLSD or $NPM1_{121-294.}$ Cells were fixed 24 h later and immunocytochemistry was performed with RFP and GFP antibodies. Viability was quantified by RFP or GFP fluorescence and DAPI staining. ***, $p<0.001$ as compared to EGFP/Ctrl transfected cells; ###, $p<0.001$ as compared to Q138-RFP/EGFP transfected cells (n=3).


A
24 h
48 h
72 h
Un
GFP
Q138-G
Q138-R
EGS
Oligomeric NPM1
Monomeric NPM1
DMSO
NPM1
Q138
GFP
B
DAPI
NPM1
Q138-G
Merge
Alive
Dead
DAPI
NPM1
Q138-R
Merge
Alive
Dead
C
Viability (%)
120
100
80
60
40
20
0
***
***
###
***
###
Un
Ctrl
GFP
NPM1
NESM
NLSD
121-294
GFP
Q138

V. DISCUSSION

In recent years, the importance of the neuronal nucleolus, the proteins that reside there, and the impact of nucleolar stress on neuronal health and during neurodegeneration have been gaining increasing attention (15). Being a key nucleolar protein and owing to its high abundance and ubiquitous expression in the brain, as well as the cellular processes it's known to regulate, NPM1 is potentially an attractive molecule in the regulation of neuronal viability. Indeed, recent evidence has suggested this (13, 38). However, there remains to be a clear consensus as to what its function in this process is. In this study, we describe a complex role for NPM1 whereby neurons require a healthy balance of its expression as too much and too little of the protein can have negative consequences. While the mechanism of death by knockdown requires additional elucidation, toxicity due to increased expression is dependent upon NPM1's ability to oligomerize and subsequently by its subcellular localization and cell cycle activation.

In proliferating cells, changes in NPM1 expression have revealed both pro-survival and pro-apoptotic qualities, depending on cellular conditions (1). Thus far, neuronal NPM1 has been investigated in two independent studies in the context of excitotoxicity, a process that leads to the necrotic death of neurons. These showed that NPM1 mRNA is decreased in glutamate treated cortical cultures from PS1 M146V mutant transgenic mice (39) and that its protein is downregulated in degenerating neurons of the rat hippocampal CA1 region as well as primary neuronal cultures following kainic acid treatment (13). We report, however, that NPM1 expression is increased in both the R6/2 transgenic and 3-NP chemical mouse models of

Huntington's disease. This increase is more specific to the striatum and observed when neurological deficits emerge. Furthermore, comparatively we find that protein levels are relatively unchanged in cultures of both HK/LK treated CGNs as well as HCA-treated cortical neurons. Thus, similar to what is seen in actively dividing cells, changes in neuronal NPM1 expression, as well as the effect this has, might be cell and context-dependent. The mode of cell death may also influence the pattern of NPM1 expression in dying neurons. Although it is possible that the upregulation of NPM1 occurs in surviving neurons, rather than dying cells, and may thus represent a neuroprotective response, results from the overexpression of NPM1 in primary neurons argue against this. Indeed, both in CGNs and cortical neurons, elevated expression of NPM1 induces death. While we do find that NPM1 can protect against mHTT toxicity in cortical neurons, this was only true when restricted to the nucleus. Wild-type NPM1 was unable to afford this protection and a cytoplasmic restriction slightly exacerbated death by mHTT. Thus more investigation is required into the *in vivo* nature of NPM1 during both early and late symptomatic stages of HD.

Treatment with two pharmacological inhibitors, co-expression with dominant negative forms of CDK1/2, and the overexpression of $p21^{Cip1/Waf1}$ all inhibit NPM1 neurotoxicity. We therefore conclude that death from NPM1 expression is the result of cell cycle activation, a process known to induce apoptosis in neurons. Activation of cell cycle proteins and evidence of abortive cell cycle re-entry has also been reported *in vivo* in various models of neurodegenerative disease (40–42). Both Cyclin E/CDK2 as well as Cyclin B/CDK1 have been reported to phosphorylate NPM1 (43, 44). It is possible that an increase in NPM1 expression results in phosphorylation by CDK1 or CDK2 thereby leading to an abortive re-entry into the cell cycle and consequently to

cell death. However, it is plausible that CDK1/2 activation is a consequence of cytoplasmic accumulation of NPM1 thereby leading to cell cycle re-entry.

While we report that ectopic expression induces death, we also find, as others (13, 38) have, that knockdown also induces neuronal death. However, while the mechanism by which knockdown of NPM1 kills neurons remains to be elucidated, this finding indicates that a physiological level of NPM1 is necessary for the survival of neurons. Interestingly, while inhibition of cell cycle machinery prevented death from forced expression in HK, only a slight protection is seen under apoptotic conditions. Thus while important for neurons under normal conditions, NPM1 might not be necessary for the regulation of death under LK. It is possible that NPM1 provides this effect under HK conditions through cooperation with a partner whose expression decreases as neurons commit to apoptosis. Alternatively, under LK conditions and the presence of elevated NPM1 expression, cell death pathways other than those regulated by cell cycle machinery could be activated.

NPM1 is typically found as higher order oligomers, the formation of which is important for its function. We investigated this fact with the use of RNA aptamers designed to interfere with oligomer formation. In line with physiological levels of NPM1 being necessary for healthy neurons, and as others have reported (25) in cell lines, inhibiting endogenous oligomerization induces death. Interestingly, when co-expressed together, NPM1 was not only no longer toxic but also fully protective against LK-induced apoptosis. The death-inducing activity of ectopically-expressed NPM1 is further blocked if it is constrained to the nucleus and we show that NPM1 was unable to oligomerize when strictly nuclear, suggesting this process required nuclear export. Less is known about monomeric NPM1. Inhibition of oligomerization by

mutation of Cys21 blocks NPM1's chaperone activity (45). Additionally, NPM1 is known to bind DNA as well as both single and double stranded RNA (46). Interestingly, it is reported to be more capable of binding DNA when there is a greater presence of monomers, whereas this is lost with greater amounts of oligomers (46). However, the mechanism by which monomeric is protective against LK- and HCA-induced apoptosis is unclear and requires further investigation. In summary, we show for the first time that the nucleolar phosphoprotein, NPM1, is selectively upregulated in the striatum of two models of Huntington's disease, the R6/2 transgenic and the 3-NP injected mice. Both knockdown and ectopic expression have detrimental effects on otherwise healthy neurons. The toxic effect from increased expression is dependent upon NPM1's ability to translocate to the cytoplasm and oligomerize, which ultimately results in cell cycle activation. However, we have found that monomeric forms of NPM1 are fully protective against the induction of apoptosis. Further, NPM1 can protect against mHTT toxicity but only in its nuclear and monomeric form. While thus far described as a protective molecule in neurons, our study sheds light on a more complicated role, thereby revealing that a more in-depth investigation is needed to fully elucidate the role of neuronal NPM1.

VI. EXPERIMENTAL PROCEDURES

Materials. Unless stated otherwise, all tissue culture media was purchased from Invitrogen (Carlsbad, CA) and chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). Poly-L-Lysine for primary neuronal cultures was purchased from Trevigen (Gaithersburg, MD). Antibodies used in this study were as follows: anti-B23 (catalog # B0556, Sigma-Aldrich), α-Tubulin (catalog # Sigma-Aldrich), β-tubulin (catalog # 5568P, Cell Signaling, Danvers, MA), Cleaved Caspase-3 (catalog # 9661S, Cell Signaling), c-Jun (catalog # 9165S, Cell Signaling), Flag (catalog # F1804, Sigma-Aldrich), GFP (catalog # SC-9996, Santa Cruz Biotechnology, Dallas, TX) and RFP (catalog # R10367, Invitrogen). Primary antibodies were used at a concentration of 1:1,000, or 1:40,000 for α-Tubulin, in 5% bovine serum albumin. HRP-conjugated secondary antibodies (from Pierce Rockford, Rockford, IL) were used at concentrations of 1:10,000 to 1:40,000.

Expression plasmids. Expression plasmids used in this study and purchased from Addgene (Cambridge, MA) are as follows: GFP- and Flag-tagged NPM1 (#17578), NESD (#13283), NESM (#13282) and NLSD (#13287) plasmids were donated by Xin Wang (22), Flag-p21 (#16240) donated by Mien-Chie Hung (47), HA-tagged dominant negative Cdc2/CDK1 (DnCDK1, #1889) and CDK2 (DnCDK2, #1885) plasmids were donated by Sander van den Heuvel (48). Mutant huntingtin (mHTT) constructs, Q138-GFP and Q138-RFP, were kind gifts from J. Troy Littleton at the Massachusetts Institute of Technology. NPM1 RNA aptamers, 1A1 and $1A1_{1\text{-}40}$ (referred to in this study as $1A1_{Trunc}$), were a kind gift from C. Yang at the Institute

of Genetics and Developmental Biology, Chinese Academy of Sciences. NPM1 mutant and deletion constructs, NoLSM and $NPM1_{121-294}$, were created by using full length NPM1 in pEGFP-C2 listed above as a template. The following primers were used to create these constructs:

Nucleolar localization signal mutant (W288G, W290G; NoLSM): NPM1fwd: 5′-TCGAATTCT-GCAGTCGAC-3′ and NoLSMrev: 5′-TCCGGTGGATCCTTAAAGAGACTTCCTCC CCTG-CCCGAG-3′. NoLSM PCR product was digested with EcorI and BamHI and then ligated into pEGFP-C2.

Oligomerization domain mutant ($NPM1_{121-294}$): $NPM1_{121-294}$fwd: 5'-CAAGGATGACGACGAC-AAGCATATGGAAGATGCAGAG-3' and NPM1-rev: 5′-TCCGGTGGATCCTTAAAGAGA-CTTCCTCCACTGCCAGAG-3′. This product was then used as a template for PCR with the primers: NPM1fwd: 5'-GGGCCCGGGATCCTGCCCGCACATGG-ACTACAAGGATGAC-3' and NPM1- rev: 5′-TCCGGTGGATCCTTAAAGAGACTTCCTCC- ACTGCCAGAG-3′. The resulting product was digested with BamHI and ligated into pEGFP-C2.

All constructs were sequenced and then transfected in HEK293T cells to check for expression by EGFP autofluorescence. Protein lysates were subjected to western blot analysis to check proper protein size.

Culture, treatment and transfection of neurons. Cerebellar granule neurons (CGNs) were cultured as previous described (49). Briefly, 7-8 day old Wistar rats were euthanized, cerebella were extracted and plated in 24-well plates ($1x10^6$ cells/well) or 60 mm dishes ($12x10^6$ cells) in culture media (Basal Minimal Eagle's medium, supplemented with 10% fetal bovine serum (FBS), 25 mM KCl, 2 mM glutamine and 0.2% gentamycin). To prevent replication of non-

neuronal cells, cytosine arabinoforanoside (10 μM) was added to the culture medium 18–22 h after plating. Transient transfections were performed on day 4-5 *in vitro* by the calcium phosphate method as previously described (50, 51) and allowed to express for 24 h (or 48 h in the case of shRNA). Cultures were then switched to serum free culture medium (Basal Minimal Eagle's medium, 2 mM glutamine and 0.2% gentamycin) supplemented with 25 mM KCl (High Potassium, HK) or without KCl (Low Potassium, LK). For pharmacological inhibitor studies, at the time of media switch cells were treated with either HK medium or HK medium supplemented with inhibitors of the following concentrations: Z-VAD at 50 μM, SB216763 at 5 μM, SP6001245 at 10 μM, Roscovitine at 50 μM or HSB13 at 25 μM. All pharmacological inhibitors were purchased from Calbiochem (Billerica, MA) and their ability to inhibit their targets at the doses listed above was confirmed in control experiments. After 24 h treatment, cells were fixed, immunocytochemistry was performed, and cell viability was quantified based on cell morphology using DAPI (4'6'-diamidino-2-phenylindole hydrochloride) staining as previously described (52, 53). Cells with condensed or fragmented nuclei were scored as dead. Co-staining for cleaved caspase-3 was used as a secondary method to confirm cell death.

Rat cortical cultures were prepared from the cerebral cortex of E16-17 Wistar rats as previously described (54–56). Cultures were maintained in Neurobasal media with 1% B27 supplement, 0.25% L-Glutamine, 1% penicillin/streptomycin, 0.1% HEPES and 1.1% sodium pyruvate without serum to minimize glial proliferation. Cultures were transfected on day 6 *in vitro* by the calcium phosphate method and allowed to express for 8 h followed by 15-16 h treatment with 1mM homocysteic acid (HCA). Viability was quantified as described above for CGNs. For cell

lysates for western blot analysis, 60 mm dishes were either left untreated or treated with 1mM HCA on day 7 *in vitro*. HCA induces death in cortical neurons through oxidative stress (57, 58).

Culture and transfection of cell lines. The HEK293T (catalog # CRL-11268) and Neuro2a (N2a, catalog # CLL-131) cell lines were purchased from ATCC and maintained in DMEM supplemented with 10% FBS. The HT22 hippocampal neuroblastoma cell line was a kind gift from Dr. Rajiv Ratan (Burke Medical Research Institute, NY) and was maintained in DMEM without sodium pyruvate supplemented with 10% FBS. All cell line transfections were performed using Lipofectamine 2000 (Life Technologies) diluted in Opti-Mem reduced serum media by following the manufacturer's guidelines.

Western blotting. Cells were lysed with 1x cell lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) containing a protease inhibitor cocktail tablet (Roche), frozen at -80°C for at least 1 h, thawed and centrifuged at 14,000 x g for 10 min at 4°C. The supernatant was collected as the whole-cell soluble lysate and protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Unless stated otherwise, 40 μg of protein was mixed with 6x sodium dodecyl sulfate (SDS) sample buffer (375 mM Tris–HCl, pH 6.8, 12% SDS, 60% glycerol, 300 mM dithiothreitol, and 0.012% bromophenol blue), boiled at 95°C for 5 min and subjected to SDS–PAGE. Proteins were electrophoretically transferred from the gel to an enhanced polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 4°C overnight. Membranes were incubated in blocking buffer (1x TBS, 5% w/v nonfat dry milk and 0.05% Tween-20) at 25°C for 1 h, then subsequently incubated at 4°C overnight with primary antibodies, which was followed by secondary antibody for 1 h at 25°C.

Immunoreactivity was developed by enhanced chemiluminescence and visualized by autoradiography using ECL or ECL prime reagents (GE Healthcare, Fairfield, CT).

shRNA-mediated knockdown. For knockdown experiments, the following shRNAs targeting NPM1 were purchased from Sigma-Aldrich: TRCN0000115427, TRCN0000115428 and TRCN0000115430 referred to here as sh1, sh2 and sh3, respectively. The pLK0.1-TRC (pLK0.1) control shRNA, which contains a non-hairpin 18 bp insert, was purchased from Addgene (#10879) donated by David Root (59). To test knockdown efficiency, each shRNA was transfected into the HT22 neuroblastoma cell line using Lipofectamine 2000 according to the manufacturer's instructions and allowed to express for 72 h. Cells were then lysed in 1x cell lysis buffer and subjected to western blotting with an antibody against endogenous NPM1. For viability studies, pLK0.1 or each shRNA was transfected into neuronal cultures along with EGFP in a 6.5:1 ratio as described above. For CGNs, cells were transfected on day 4 *in vitro*. Medium was switched 48 h later to HK/LK media for 24 h. Cortical neuronal cultures were transfected on day 6 *in vitro* and allowed to express for 72 h. Cells were then fixed and DAPI stained and viability was quantified based on EGFP fluorescence.

Crosslinking analysis. Crosslinking was performed as previously described (60, 61). HEK293T or N2a cells were transfected using Lipofectamine 2000 and allowed to express for 24-72 h. Cells were then lysed in 150 μL of HEGNT buffer **(**20 mM HEPES pH 7.5, 1 mM EDTA, 10% glycerol, 0.4 M NaCl, 1% Triton X-100**)** by freezing at -80°C for at least 1 h, thawed and centrifuged at 14,000 x g for 10 min. 50 μg of whole cell lysate was then incubated with either DMSO or 0.5 mM ethylene glycol bis-succinimidylsuccinate (EGS) for 30 min at 25°C. Crosslinking was quenched with the addition of 0.025 mM Tris-HCl, pH 7.5 for 15 min at 25°C.

Reactions were then boiled in 6x SDS at 95°C for 5 min and then subjected to SDS-PAGE on an 8% gel.

The R6/2 transgenic mouse model of HD. Female mice hemizygous for an ovarian transplant of exon 1 of the human huntingtin transgene containing 120 +/- 5 CAG repeats were bred with wild-type (WT) B6CBAF1/J male mice (Jackson Laboratory). Genotyping was performed 5-7 days after birth following IACUC approved guidelines. For subsequent generations of breeding, transgenic (R6/2) male mice were bred to non-littermate WT females. At 6, 10 and 12 weeks of age, gender matched WT and R6/2 littermates were euthanized and brains were dissected into the following regions: striatum (STR), cortex (CTX), cerebellum (CBM) and the rest of the brain (other brain parts, OBP). Tissue was homogenized in 1x RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin) containing a protease inhibitor cocktail tablet and lysed by freezing at -80°C for at least 1 h. Samples were then thawed, centrifuged at 14,000 x g for 10 min and subjected to western blotting.

The 3-NP mouse model of HD. Ten week-old C57BL/6 male mice (Charles River Laboratories, Wilmington, MA) were administered 3-nitropropionic acid (3-NP) as previous described (30, 62, 63). Briefly, mice received 10 intraperitoneal injections of either saline control (ctrl) or 3-NP (50 mg/kg, pH 7.4) every 12 h for 5 days. Pairs of control (C) and 3-NP mice were euthanized by CO_2 inhalation after one day (D1), three days (D3) or five days (D5) of injections. Brains were dissected into either the striatum (STR) or the rest of the brain (other brain parts, OBP). The tissue was then homogenized in 1x RIPA buffer and lysed by freezing at -80°C for at least 1

h. Samples were thawed, centrifuged at 14,0000 x g for 10 min and subjected to western blotting.

Animal usage. All procedures conducted using animals were reviewed and approved by the Southern Methodist University Institutional Animal Care and Use Committee (IACUC).

Statistical analysis. All graphs were created and statistical analysis was performed using the GraphPad Prism software and all densitometric analysis was done using ImageJ. Student's *t*-test was performed for statistical analysis. For comparing multiple datasets, one-way ANOVA with Tukey's multiple comparisons posttest was used. Results are shown as mean ± standard deviation from at least three independent experiments. *p* values of $p<0.05$ were deemed statistically significant. Asterisks were used to denote statistical significance: * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. Unless mentioned otherwise, all viability experiments were performed in duplicate and repeated three times. For each viability experiment, ≥200 transfected cells were counted.

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CHAPTER 4

PROTECTION AGAINST NEURONAL APOPTOSIS BY NAD^+-DEPENDENT SIRTUIN FAMILY MEMBERS SIRT1 AND SIRT5

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II. ABSTRACT

The primary focus of this dissertation centers on a protein that we identified in a mass-spectrometric screen for interacters of the Class III HDAC, SIRT1. In addition to studying the nature of how NPM1 regulates neuronal viability, work has also been performed in examining the role of SIRT1 in neuroprotection. Further, we have also performed some work on SIRT5, another member of the sirtuin family. Previously published research from our laboratory on the sirtuins revealed that, of the seven, only SIRT1 and SIRT5 were protective against LK-induced apoptosis of CGNs. Through the use of pharmacological inhibition against its deacetylase activity, as well two different deacetylase-dead point mutants defective in NAD^+ binding, we showed that SIRT1 is able to confer a neuroprotective effect in a deacetylase-independent manner. This protection was further extended to HT22 cells induced to die by both serum deprivation and HCA treatment. While known as one of the three mitochondrial SIRTs, ectopic expression of SIRT5 revealed a nuclear and cytoplasmic localization and protection of CGNs from death. However, in the few cells that were dead, SIRT5 was found to localize to the mitochondria. Interestingly, this protective effect was not extended to HT22 cells. Indeed, SIRT5 induced death in otherwise healthy HT22 cells and exacerbated death under HCA treatment. The focus of this chapter is to elaborate on these previous findings with new data and provide insight to elucidate the potential mechanisms by how SIRT1 and SIRT5 may regulate the survival of post-mitotic neuron.

III. INTRODUCTION

Mammals and other higher eukaryotes express eighteen proteins that together comprise a class of enzymes known as histone deacetylases (HDACs). These proteins were initially characterized by their role in chromatin remodeling in which they function by removing acetyl groups from N-terminal tails of lysine resides on histones, thereby resulting in chromatin condensation and thus a decrease in transcription. It is now known that apart from histones, HDACs interact with and regulate a wide array of other proteins, which in terms of neuronal function can impact the viability of neurons.

Based on their sequence homology to the yeast proteins from which they were discovered, HDACs are categorized into four classes: Class I (HDACs 1, 2, 3 and 8) are homologues of the yeast Rpd3, Class II (HDACs 4, 5, 6, 7, 9, 10) are homologues of the yeast Hda1 protein, Class III (the sirtuins, SIRT1-7) are homologues of the yeast Sir2 and finally Class IV (HDAC 11), which has little sequence homology to either Rpd3 or Hda1 (1). Classes I, II and IV are collectively known as classical HDACs based on their need of a molecule of Zn^{2+} to catalyze the removal of acetyl groups. The activity of these HDACs can be inhibited by compounds (HDACi's) such as trichostatin A (TSA) that bind to their Zn^{2+} containing catalytic domain. Sirtuins, however, are considered non-classical HDACs because while they do retain HDAC activity, this is instead dependent on NAD^{+}. Furthermore, the catalytic activity of sirtuins is not inhibited by compounds that target classical HDACs but are blocked pharmacologically by others such as nicotinamide, Sirtinol and Ex-527.

HDACs were originally identified as potential mediators of neuronal viability based on the finding that HDACi's are protective in models of neurodegeneration (2). As these inhibitors are broad spectrum and thus target all classical HDACs, elucidating the roles of individual HDACs on the health of the neuron became an important task. Since then it has found that some Class I and II HDACs do indeed have important roles in either protection from or instigation of neuronal death (3–9). However, as sirtuins are not targeting by these inhibitors, their roles remained elusive. While it was known that SIRT1 and SIRT2 contributed protective and toxic effects, respectively (10), it was unclear what effect, if any, the rest of these seven proteins have on the health of neurons. Though we have delineated potential roles of each sirtuin member (11), as the focus of this chapter is on SIRT1 and SIRT5, the effects we have reported of the other five will only be briefly mentioned.

SIRT1, originally known as Sir2α, was the first mammalian sirtuin discovered and has the highest sequence similarity to the yeast Sir2 protein. It is a heavily studied molecule with implications in a range of biological processes including DNA damage, oxidative stress, apoptosis, cell proliferation, transcriptional silencing and circadian rhythm (12–14). While it has shown cytoplasmic shutting in response to oxidative stress (15), SIRT1 is largely a nuclear protein where it deacetylates histones H3 and H4 and interacts with an array of non-histone proteins including, but not limited to, NF-κB, FOXOs, Ku70, p53, PARP1, p300, PGC1-α, AMPK, DBC1, E2F1 and NPM1 (16–22). Further, SIRT1 has displayed protective effects in both cell culture and mouse models of neurodegenerative diseases including Alzheimer's disease and amyotrophic lateral sclerosis as well as α-synuclein and polyglutamine toxicity (23–26). Apart from its role in positively regulating the survival of post-mitotic neurons, SIRT1 has

additionally been implicated as both a tumor promoter and suppressor. Indeed its expression has been observed as upregulated in some cancers and downregulated in others (27). Through its interactions with proteins such as Ku70, PARP1 and p53, SIRT1 is known to regulate the cellular response to DNA damage and thus the G_1/S transition of the cell cycle. Its mRNA is positively regulated by BRCA1, a protein that works in conjunction with PARP1 and regulates DNA repair (28). Additionally, two proteins involved in activation of p53-mediated apoptosis, HIC1 and DBC1, target and inhibit SIRT1 (29, 30). All of these interactions, along with those of E2F1 and NPM1, two proteins critical of cell proliferation, provide support that SIRT1 regulates the cell cycle's exit out of G_1 and into S-phase. However, if this role extends to neurons and protection against neurodegeneration is less clear.

While SIRT1 is extensively studied, much less is known on SIRT5. It is known to possess deacetylase activity, albeit reported as weak, but additionally has demalonylase and desuccinylase activity (31). Perhaps its most well-investigated role to date is in the liver mitochondria matrix where it deacetylates carbamoyl phosphate synthetase (CPS1), the rate-limiting step of the urea cycle, thereby stimulating its activity (32, 33). SIRT5 knockout mice display increase blood ammonia levels during fasting (33). It has also been shown to shift to the mitochondrial intermembrane space and deacetylate cytochrome c, a critical intermediate in the induction of apoptosis (34). Of the little that is known about neuronal SIRT5, much has centered on expression levels. It has been suggested that a polymorphism in its promoter may represent a risk factor for mitochondrial dysfunction-related neurological diseases (35). Further, increased SIRT5 mRNA was found in gray matter of the spinal cord of human postmortem ALS patients (36). Similarly, its protein expression increases during the progression of AD, although this may

be related to its appearance in activated microglia (37). Subcellular fractionation of the postmortem AD tissue showed SIRT5 present in nuclear, cytoplasmic and mitochondrial fractions (37). Mice lacking the pre-synaptic serotonin$_{1B}$ (5-HT$_{1B}$) receptor show early age-related motor decline, decreased longevity and have increased SIRT5 transcripts levels in the cortex and striatum (38). While these increases in expression might imply a contribution to disease progression, other evidence suggests the contrary. SIRT5 knockout mice exposed to MPTP, a chemical model of PD, showed more severe nigrostriatal dopaminergic degeneration compared to wild-type controlsl (39). Further, in models of cerebral ischemia, PKCε activation increased SIRT5 expression and desuccinylase activity in a Nampt-dependent manner and protected against metabolic and ischemic stress (40). This PKCε protection failed to occur in SIRT5 knockout mice (40). Interestingly, humans express two SIRT5 isoforms that are identical in their first 285 amino acids but differ slightly in their C-termini and show different localization patterns (41). While isoform 1 (310 a.a.) shows a mitochondrial and cytoplasmic localization and is stabilized in the cytoplasm by a GPCG-motif in its C-terminus, isoform 2 (299 a.a.), which is found only in primates, is strictly mitochondrial (41). This subcellular localization difference is due to the fact that while both contain a cleavable mitochondrial-targeting signal at their N-termini, isoform 2 contains a mitochondrial membrane insertion signal at its C-terminus (41). Thus these findings, in conjunction with our results elaborated on later in this chapter, reveal that as with many proteins, SIRT5's ability to instill neuroprotection might be cell type and context-specific. However, it is unclear which isoform is increased in the diseased tissues just discussed. As it is expressed as two nearly identical isoforms that show distinct localization patterns, it is possible that they also exhibit distinct abilities to regulate neuronal survival.

IV. THE ROLE OF SIRT1 IN NEURONAL SURVIVAL

As our laboratory began investigating the roles of individual HDACs, apart from SIRT1 and SIRT2 little was known about the neuronal sirtuins. An initial survey of each SIRT expressed in CGNs under HK/LK treatment revealed that while SIRT4 and SIRT7 had no effect, the other five had an influence on the health of the neurons (11). As previously shown, and expected, SIRT1 was protective against and SIRT2 induced death in otherwise healthy neurons (11). Of the remaining three, SIRTs 3 and 6 were additionally toxic under HK treatment and further exacerbated death under LK, but SIRT5 was protective against LK-induced apoptosis (11). Further evaluation of SIRT1 using pharmacological treatments of nicotinamide and sirtinol, which are known to target SIRT1 deacetylase activity, as well as two different deacetylase-dead point mutant constructs, suggested that SIRT1 was providing its protective effect in a deacetylase-independent manner (11). We have subsequently found that this protection extends to cortical neurons induced to die by HCA treatment (Fig. 4.1).

With these findings being the result of overexpression-based experiments, we first wanted to determine if SIRT1 was necessary for the viability of normal healthy neurons. This was accomplished through shRNA-mediated knockdown. Commercially available shRNAs were purchased and first tested for their efficacy in knocking down SIRT1 expression. As shown in figure 4.2A, sh-1 and sh-2 reduced SIRT1 protein level. When expressed in CGNs, both sh-1 and sh-2 induced death in otherwise healthy neurons kept alive by HK treatment (Fig 4.2B). While sh-2 had no effect on LK-treated cells, sh-1 showed an increase in toxicity beyond that of

the pLK0.1 control vector. Sh-3, which showed no knockdown, had no effect on the viability under both HK and LK (Fig 4.2A and B).

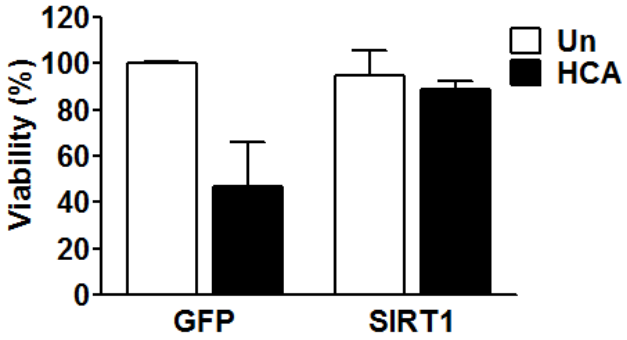


Figure 4.1. SIRT1 protects cortical neurons from HCA-induced death. Cortical cultures were transfected with either GFP or SIRT1 for 8 h. Cells were then treated with 1 mM HCA for 15-16. Viability was quantified by immunocytochemistry with GFP or Flag antibodies and DAPI staining (n=2).

In order to elucidate the mechanism by how SIRT1 is protecting neurons we sought to narrow the region within SIRT1 conferring this effect. We obtained ten different constructs, referred to here as Δ1-10, that contain ~70 sequential amino acid deletions spanning the SIRT1 protein from Dr. Zhenkun Lou's laboratory at the Mayo clinic (42) (Fig. 4.3). Consistent with our previous pharmacological and mutant data, expression of these constructs into CGNs showed that deletion of any part of the catalytic domain (Δ4-7) had no effect on viability compared to their wild-type counterpart (Fig 4.4A). However, it was revealed that Δ8, a 67 amino acid region just C-terminal to the catalytic domain, is responsible for providing SIRT1's protection (Fig 4.6A). Interestingly, it has been reported that a 25 amino acid region in SIRT1's C-terminal (a.a. 631-655), which has been termed ESA (essential for SIRT1 activity), is required for SIRT1 activity (43). This ESA region will interact with SIRT1's catalytic domain acting as an on-switch and

thus without it SIRT1 is catalytically dead (43). As shown in Figures 4.3 and 4.4A, the ESA is located within Δ9 and expression of this construct was just as protective against LK-induced apoptosis as wild-type SIRT1. Interestingly, the Δ1 construct is strictly cytoplasmic but still retains its protective ability (Fig. 4.4B). These results confirmed that SIRT1's neuroprotective effect is indeed by a deacetylase-independent mechanism that occurs through Δ8 (a.a. 542-609). As SIRT1 is protective in models of neurodegeneration, we investigated if it could also protect against mutant huntingtin toxicity independent of its catalytic activity. We have previously reported that while wild-type huntingtin (Q15) does not induced death in CGNs, mutant

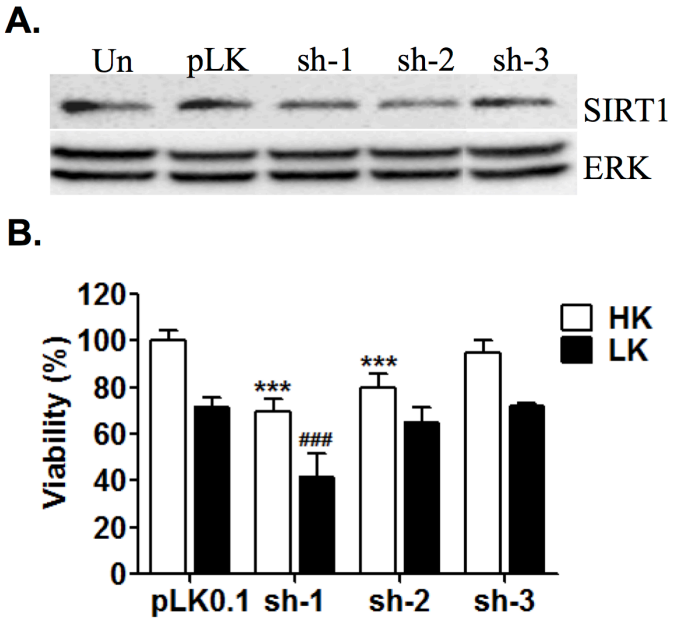


Figure 4.2. Knocking down Sirt1 induces death in CGNs. *A.* HT22 cells were either left untransfected (Un) or transfected with a pLK0.1 control, sh-1, sh-2 or sh-3 for 72 h. Cells were then lysed and western blotting was performed and probed for endogenous SIRT1. ERK1/2 serves as a loading control. *B.* CGNs were transfected with a pLK0.1 control or the three shRNAs along with GFP in a 6.5:1 ratio. 48 h later media was switched to HK/LK for 24 h. Viability was quantified by GFP fluorescence and DAPI staining. ***, $p<0.001$ as compared to pLK0.1 HK. ###, $p<0.001$ as compared to pLK0.1 LK (n=3).

huntingtin (Q138) does (4) (Fig. 4.5). When Q138 is expressed into CGNs along with a control vector (pLK0.1), it is still toxic (Fig. 4.5). However, when expressed along with wild-type SIRT1, a deacetylase dead SIRT1 (H363Y), Δ's 1, 4, 6, 7 and 9, toxicity was rescued (Fig. 4.5). Interestingly and consistently, Δ8 was unable to provide this protection (Fig. 4.5)

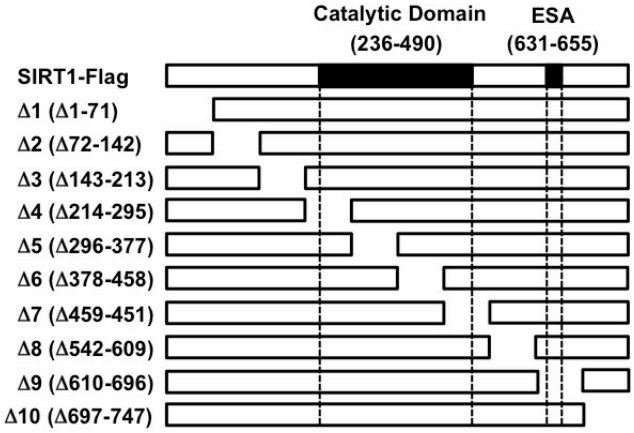


Figure 4.3. Schematic of the SIRT1 deletion constructs. Ten different flag-tagged SIRT1 deletions constructs spanning the SIRT1 protein and the wild-type construct from which they were created.

Dependence on classical HDACs

Two potential mechanisms influencing the Δ8 region and SIRT1-mediated survival are regulation by a pro-survival signaling pathway and protein-protein interaction. We first explored the idea that SIRT1 may be post-translationally modified by phosphorylation through pharmacological inhibition. Targeting of pro-survival signaling cascades though inhibition of MEK, PI3-K, AKT, CK1, PKA or CaMK II had no effect on SIRT1 (Fig. 4.6). However, treatment with two different classical HDAC inhibitors blocked SIRT1- mediated protection (Fig. 4.6). As neither of these should target SIRT1 activity, this suggested that SIRT1 protection is dependent on a classical HDAC. While TSA is a broad-spectrum HDAC inhibitor, the other

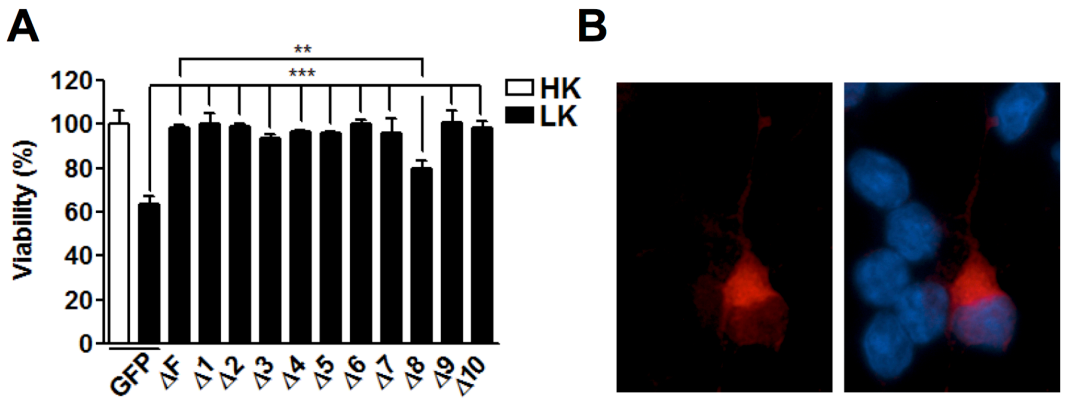


Figure 4.4. SIRT1-mediated protection is dependent on the Δ8 region. *A*. GFP, SIRT1 and Δ1-10 were transfected into CGNs and treated 24 h later with HK or LK media for an additional 24 h. Viability was quantified by immunocytochemistry with a GFP or Flag antibody and DAPI staining. **, $p<0.01$; ***, $p<0.001$ (n=2). B. Localization of ectopically expressed Δ1 into CGNs.

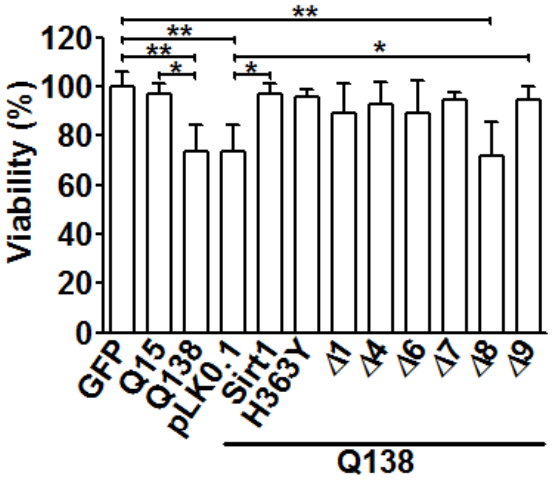


Figure 4.5. SIRT1 protects against mut-HTT in a deacetylase-independent manner. CGNs were transfected with GFP, Q15, Q138 or Q138 co-transfected in a 1:2 ratio with either a control (pLK0.1), SIRT1, SIRT1-H363Y, Δ1, Δ4, Δ6, Δ7, Δ8 or Δ9 for 24 h. Cells were then treated with HK media for another 24 h. Viability was quantified by immunocytochemistry with a GFP antibody and DAPI staining. Co-transfections were counted based on Q138 expression. *, $p<0.05$; **, $p<0.01$ (n=3).

used here, HDACi, targets Class I HDACs (1, 2, 3 and 8), of which it is specific to HDACs1, 2 and 3 and less so to HDAC8 (44). Recently, our lab has published that HDAC3 is highly and selectively toxic to neurons and HDAC1 can be either protective or toxic depending on if it interacts with the truncated form of HDAC9 (HDRP) or HDAC 3, respectively (3, 5). These findings suggested that we turn our attention to HDAC1.

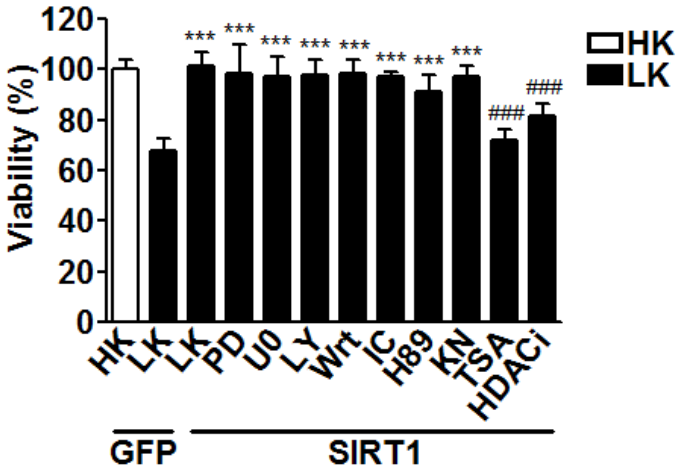


Figure 4.6. SIRT1 protection is blocked by classical HDAC inhibitors. GFP or SIRT1 were transfected into CGNs for 24 h. Media was the switched to either HK, LK or LK supplemented with the following inhibitors for 24 h: U0126 (MEK1/2, 10 μM), PD98059 (MEK1, 50 μM), Wortmannin (PI3-K, 100 nM), LY294002 (PI3-K, 10 μM), H89 (PKA, 3μM), KN-62 (CamKII, 10 μM), Nicotinamide (5 mM), Sirtinol (100 μM), TSA (HDACs 1 μM), Pimelic Diphenylamide 106 (HDACi 10 μM). Viability was quantified by immunocytochemistry with GFP or Flag antibodies and DAPI staining. ***, $p<0.001$ as compared to GFP LK; ###, $p<0.001$ as compared to SIRT1 LK (n=3).

We first examined if the two proteins interacted by immunoprecipitation following co-expression of HDAC1 with either HDAC3 or SIRT1 into HEK293T cells. As can be seen in figure 4.7A and as previous described, HDAC1 successfully immunoprecipitated with HDAC3. As expected, albeit to a lesser extent, HDAC1 was additionally pulled down by SIRT1 (Fig 4.7A).

Since Δ4-7 and Δ9 are important for SIRT1's deacetylase activity and Δ8 is important for neuroprotection, we further investigated if the interaction with HDAC1 is altered by the removal of these regions. Interestingly, an interaction was greatly reduced, if present at all, with Δ8 (Fig 4.7B). Furthermore, while no change from wild-type SIRT1 is seen with Δ4-7, Δ9, which lacks the ESA, showed a greater interaction with HDAC1.

Increased expression of HDAC1 alone induces death in otherwise healthy CGNs through cooperation with HDAC3, but has the potential to be protective if there is interference with this interaction (3). We tested the idea that SIRT1 may block HDAC1 toxicity. Indeed, both wild-type SIRT1 and Δ9 rescued HDAC1-induced death (Fig. 4.8A). Further, as expected since they failed to interact, Δ8 was unable to provide this effect (Fig. 4.8A). Interestingly, a deacetylase-deficient HDAC1, which has its first 56 amino acids deleted and termed HDAC1-Δ56, is as toxic as wild-type HDAC1 because it can still interact with and use HDAC3's deacetylase activity (3). Similar to wild-type HDAC1, when co-expressed together, wild-type SIRT1 and Δ9 can block this toxicity but Δ8 cannot (Fig 4.8B). This suggests that HDAC1 may not need to be in an active form to interact with SIRT1. Indeed, following co-transfection of SIRT1 or Δ8 with HDAC1-Δ56 into CGNs, immunocytochemical analysis showed a decrease in co-localization between Δ8 and HDAC1-Δ56 (Fig 47C). Further, co-immunoprecipitation experiments suggest that SIRT1 interacts with HDAC1-Δ56 in the same manner as full length HDAC1 (data not shown). While these overexpression results point to an importance of HDAC1 and SIRT1 cooperation in protection, as HDAC inhibitors are not targeting SIRT1, blockage of protection by their treatment suggest the necessity of endogenous HDAC(s). As such, we investigated if

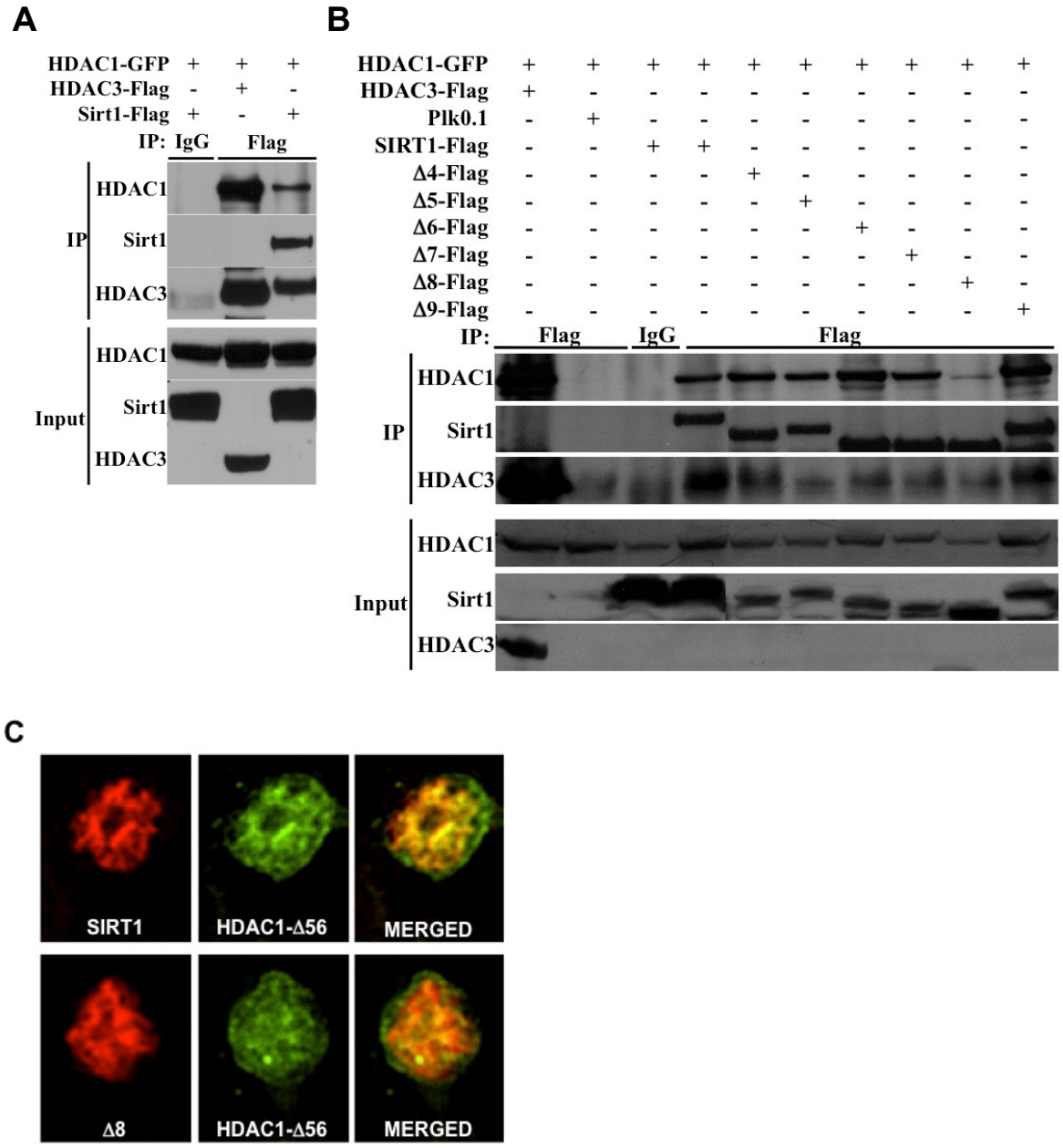


Figure 4.7. An HDAC1 and SIRT1 interaction is dependent on Δ8. *A and B.* HEK293T cells were transfected as follows: (A) HDAC1-GFP and either HDAC3-Flag or SIRT1-Flag; (B) HDAC1-GFP and either a control vector (pLK0.1), HDAC3-Flag, SIRT1-Flag or Δ4-Δ 9-Flag. Immunoprecipiation was performed by pulling down with a Flag or IgG antibody and then subjected to western blotting. Blots were probed with a GFP antibody and then reprobed for Flag. *C.* Representative images showing co-localization of CGNs co-transfected with either SIRT1 and HDAC1-Δ56 (top) or Δ8 and HDAC1- Δ56 (bottom).

knocking down endogenous HDAC1 interfered with SIRT1 protection. Co-expression with shRNAs targeting endogenous HDAC1 previously shown to knockdown the protein (3) revealed that SIRT1 is unable to protect against LK-induced apoptosis without HDAC1 (Fig 4.8 C). These results suggest that SIRT1 is able to provide deacetylase-independent neuroprotection through an interaction with HDAC1 within the Δ8 region. However, while SIRT1 requires HDAC1 present to protect neurons, knowing that SIRT1 acts in a similar manner with a deacetylase-deficient HDAC1 lends the idea that the story does not end there. HDACs are known to work as part of bigger complexes, both with each other as well as with other non-HDAC proteins. Based on the inhibitor data, deacetylase activity of a Class I HDAC appears necessary for SIRT1 protection. As HDAC1 is the only Class I HDACs investigated thus far with SIRT1, it is possible that SIRT1 forms a complex with HDAC1 and an as-of-yet investigated HDAC. With HDAC3 being highly toxic to neurons as well as localizing to both the cytoplasm and nucleus, it does not seem as a prime candidate, thus leaving HDACs2 and 8. As the HDACi used against SIRT1 is less specific for HDAC8 and since HDAC2 is similar in nature to HDAC1, it is possible that a complex is formed between SIRT1, HDAC1 and HDAC2. However, more investigation is required to fully elucidate the exact nature by how SIRT1 protection is dependent on classical HDAC(s).

Regulation of cell cycle induction through Δ8

While inhibitor work led down a path towards investigating the involvement of classical HDACs, we concurrently considered SIRT1 protection was awarded through protein-protein interaction independent of deacetylase activity. Potential candidates were discovered through mass-spectrometry. Following overexpression of GFP and Flag-tagged SIRT1 and Δ8 constructs

into HT22 cells, they were immunoprecipitated with a Flag antibody and mass spectrometry was performed to identify candidates that interacted with full length SIRT1 but not Δ8. Three proteins, protein phosphatase 1A (PP1A), ZNF346 (also know as JAZ) and nucleophosmin 1 (NPM1) were identified as potentially attractive.

While PP1A has yet to be investigated, work has been published from our laboratory showing that the zinc finger JAZ is necessary for neurons and protects both cerebellar granule and cortical neurons against the induction of apoptosis (45). This is accomplished by inhibiting the cell cycle through the induction of $p21^{Cip1/Waf1}$ protein expression (45). As described in chapter 2 above, NPM1 can be viewed as a master regulator of sorts of the cell cycle in proliferative cells. Further, in chapter 3 I describe that in neurons its increased expression, cytoplasmic localization and ability to oligomerize induces cell cycle progression and the death of neurons. As a result of these two reports, some work has been performed examining a relationship between SIRT1 and the cell cycle in neurons.

Figure 4.4 shows that all of the SIRT1 constructs, save Δ8, are protective against LK-induced death. Further inquiry into Δ8 revealed that not only is it not protective under apoptotic conditions, it is slightly toxic to otherwise healthy neurons kept alive by HK treatment (Fig 4.9A). As both JAZ and NPM1 regulate cell cycle progression, we inquired if Δ8 could be rescued by inhibiting the cell cycle. Inhibition of the cell cycle with three different CDK inhibitors rendered Δ8 as protective against LK-induced apoptosis as wild-type SIRT1 (Fig. 4.9B). To further investigate a link to cell cycle progression, BrdU labeling experiments were performed in HT22 cells. BrdU, or bromodeoxyuridine, is a synthetic analog of thymidine that is taken up by the cell and incorporated into DNA as the cell passes through S-phase. This newly

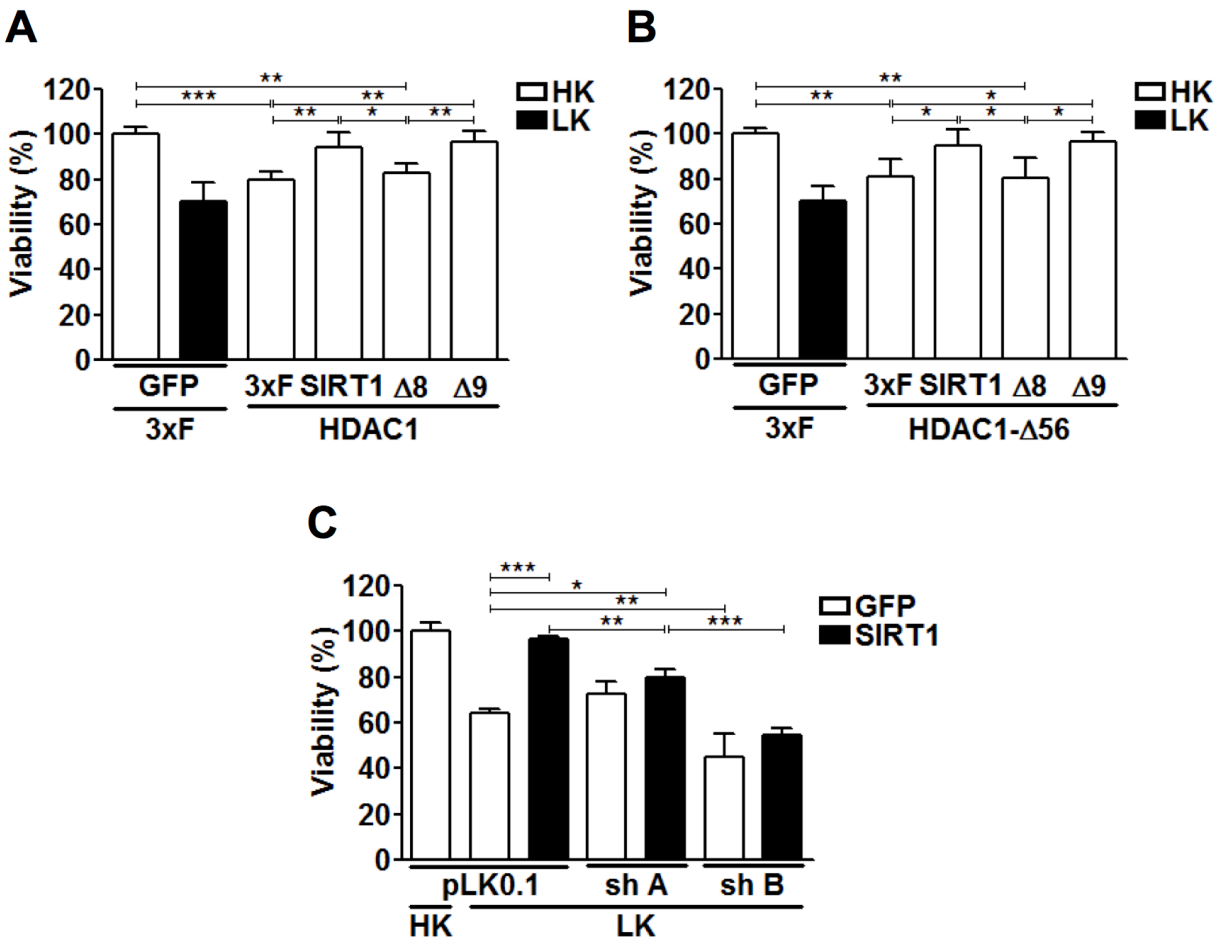
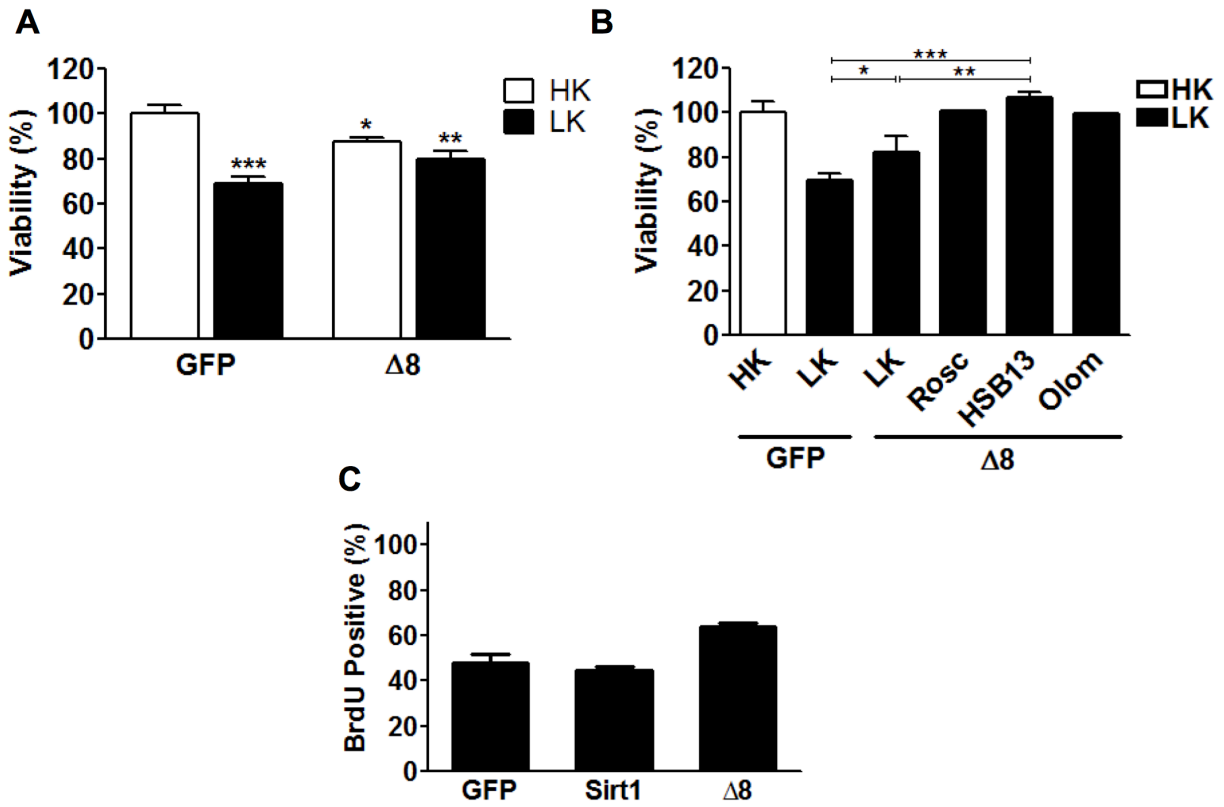


Figure 4.8. Effect of SIRT1 on HDAC1-mediated death. *A*. CGNs were transfected with either GFP and a control vector (3xFlag, 3xF) or GFP-tagged HDAC1 and 3xF, SIRT1, Δ8 and Δ 9 in a 1:2 ratio for 24 h. Cells were then treated with HK/LK media for 24 h. Viability was quantified by immunocytochemistry and DAPI staining based on GFP or HDAC1. *, $p<0.05$; **, $p<0.01$ (n=3). *B*. The same transfected was performed as in (A) but with HDAC1-Δ56. *, $p<0.05$; **, $p<0.01$; ***$p<0.001$ (n=3). *C*. GFP and SIRT1 were co-transfected into CGNs with pLK0.1 or two shRNA targeting HDAC1 in a 1:2 ratio for 48 h followed by a 24 h HK/LK treatment. Viability was quantified by immunocytochemistry and DAPI staining with a GFP or Flag antibody based on GFP or SIRT1 transfection *, $p<0.05$; **, $p<0.01$; ***$p<0.001$ (n=3).

incorporated analog can then be labeled with a BrdU-specific antibody to recognize actively proliferating cells. While wild-type SIRT1 only slightly inhibited the cell cycle compared to the GFP control, Δ8 increased the proliferation rate by ~15% (Fig. 4.9C). Together, these results

suggest that SIRT1 may protect against neuronal apoptosis though the Δ8 region by regulating aberrant cell cycle re-entry.

Apart from our mass-spectrometry data, only one study has described a relationship between SIRT1 and NPM1, where NPM1 is deacetylated by SIRT1 in oral squamous cell carcinoma (20). Since ectopic NPM1 expression leads to cell cycle activation and death in healthy neurons, we examined if SIRT1 could block this toxicity. As figure 4.10 shows, wild-type SIRT1 did indeed significantly inhibit NPM1-induced death, a finding that was further seen with Δ1, the cytoplasmic SIRT1 construct. While Δ8 provided some protection (Fig. 4.10), it was not greater than the viability of Δ8 alone (Fig 4.9 and 4.10). Protection, however, was not significantly awarded by co-expression with Δ5 but was with Δ9 (Fig. 4.10). Preliminary co-



Previous Page: Figure 4.9. Δ8 is rescued by cell cycle inhibition. *A.* CGNs were transfected with either GFP or Δ8 for 24 h. Medium was then switched to HK/LK media for 24 h. Viability was quantified by immunocytochemistry and DAPI staining with GFP or Flag antibodies. **p*<0.05, ***p*<0.01, ****p*<0.001 as compared to GFP HK (n=3). *B.* CGNs were transfected with either GFP or Δ8 for 24 h. Medium was then switched to either HK, LK or LK supplemented with roscovitine (Rosc, 50 μM, n=1), HSB13 (25 μM, n=2) or Olomeucine (Olom, 100 μM, n=1) for 24 h. Viability was quantified as done in (A). **p*<0.05, ***p*<0.01, ****p*<0.001. *C.* HT22 cells were transfected with GFP, SIRT1 or Δ8 for 22 hours and then treated with BrdU (20 μM) for 2 hours. Proliferation was quantified by co-staining with either GFP or Flag and Brdu antibodies (n=2).

immunoprecipitation data has indicated that the interaction between NPM1 and SIRT1 is lost by deletion of the Δ5 and Δ8 regions (data not shown). While Δ5 encompasses the area responsible for NAD^+ binding, thus making the construct deacetylase dead, Δ9, which contains the ESA, protects as well as wild-type SIRT1 (Fig 4.10). It is possible that the Δ5 region merely serves as a docking site between the two proteins and a deacetylase-independent mechanism is still responsible for this protection. However, the nature of this protection remains unclear and requires further elucidation.

Interestingly, both HDAC1 and 2 have previously been shown to regulate cell cycle progression and exit (46–48). Thus as HDAC1 and potentially HDAC2 are required for SIRT1-mediated protection, the complex may work together to block exit from G_0 and entrance into G_1.

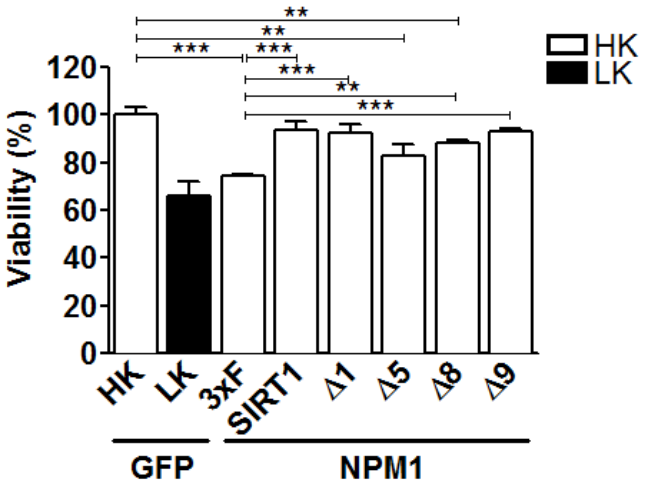


Figure 4.10. SIRT1 blocks NPM1-induced death. CGNs were either transfected with GFP or co-transfected with NPM1 and a control vector (3xF), SIRT1, Δ1, Δ5, Δ8 or Δ9 in a 1:2 ratio for 24 h. Cells were then treated with either HK or LK media for an additional 24 h. Viability was quantified by immunocytochemistry based on GFP or NPM1 fluorescence and DAPi staining. $^{**}p<0.01$, $^{***}p<0.001$ (n=3; except Δ5, n=2)

V. THE ROLE OF SIRT5 IN REGULATING NEURONAL APOPTOSIS

SIRT5, along with SIRTs 3 and 4, comprise the mitochondrial sirtuins that had yet to be examined in regard to neuronal viability. As described above, while SIRT4 had no effect on the health of the neuron, SIRT3 induced death under both pro-survival and pro-apoptotic conditions while SIRT5 protected against it. Interestingly while ectopically expressed SIRTs 3 and 4 were mitochondrial, SIRT5 showed a dual localization pattern (11). While found mitochondrial in dead cells, SIRT5 predominately displayed a nuclear and cytoplasmic localization and here promoted cell survival (11). While protective against LK-induced death in CGNs, it was unable to block death of cortical neurons against oxidative stress induced by HCA treatment (Fig 4.11). This, however, is not surprising because as described above, SIRT5 has been shown to interact with cytochrome c, whose release from the mitochondria upon DNA damage can trigger the induction of apoptosis. Interestingly, we previously reported that in the HT22 cell line, SIRT5 induced death under both untreated and serum free conditions and exacerbated the death due to HCA treatment (11). Analysis of endogenous SIRT5 mRNA in CGNs showed a decrease in cells induced to die by LK-treatment, suggesting an importance for healthy neurons (Fig 4.12A). However, in HT22 cells treated with HCA SIRT5 revealed an increase in its expression 9 h after treatment as compared to 9 h without it (Fig 4.12B). Further, in the 3-NP chemical model of HD, its mRNA is decreased in the striatum after 3 days of treatment when cell death is robust (Fig 4.12C). Thus, owing to its relationship with the mitochondria, SIRT5's ability to regulate neuronal viability may be context-and stimulus-dependent.

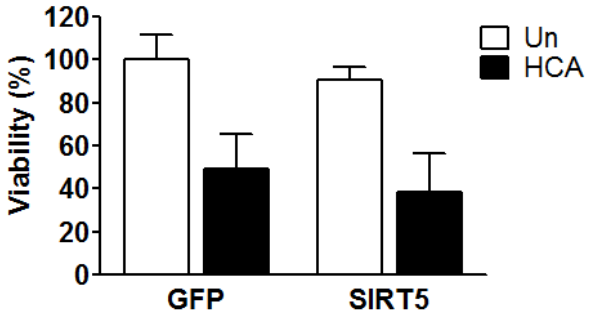


Figure 4.11. SIRT5 is unable to rescue cortical neurons from HCA-induced death. Cortical neurons were transfected with either GFP or SIRT5. Cells were treated 8 h later with 1 mM HCA for 15-16 h. Viability was quantified by immunocytochemistry with GFP or Flag antibodies and DAPI staining (n=2).

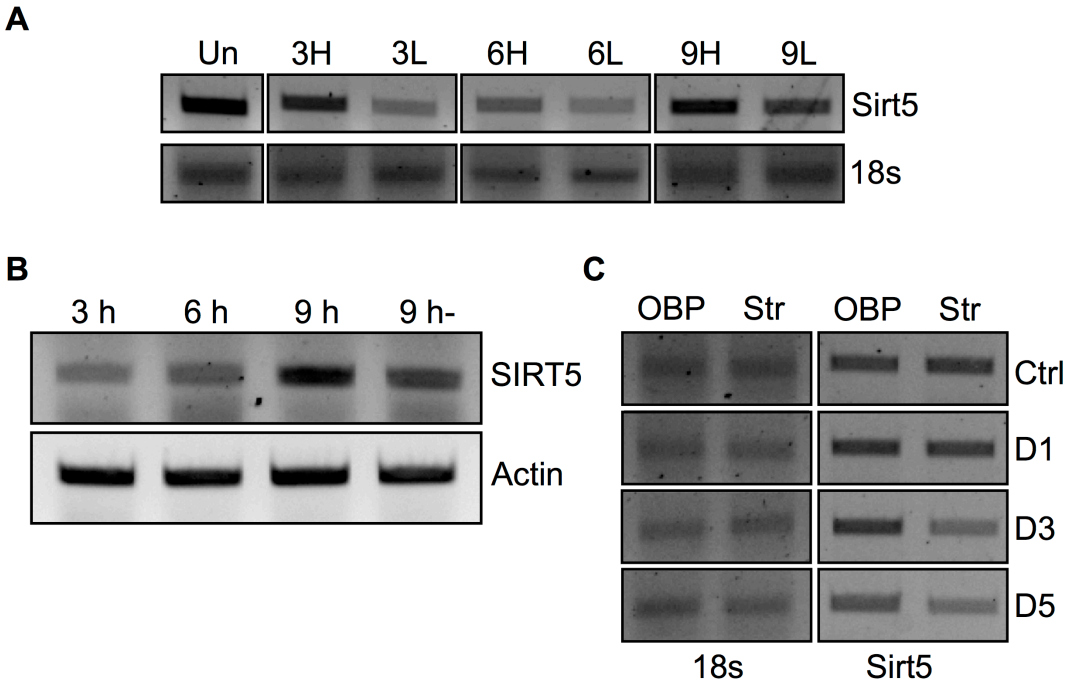


Figure 4.12. SIRT5 mRNA expression in cell culture models of apoptosis. *A.* CGNs cultures were treated with either left untreated (un) or treated with HK/LK media on day 7 *in vitro* for 3, 6 and 9 hours. mRNA was then extracted and RT-PCR performed. 18s serves as a normalization control. *B.* HT22 cells were then treated with 1mM HCA for 3, 6 and 9 h or left untreated for 9 h (9 h-). mRNA was extracted and RT-PCR was performed. Actin serves as a normalization control. *C.* mRNA was extracted from mice injected with either saline (Ctrl) or 3-NP for 1 day (D1), 3 days (D3) or 5 days (D5) as described in chapter 3 and RT-PCR was performed.

The pro-survival nature SIRT5 displays in CGNs compared to the pro-apoptotic effects seen in a proliferative cell line indicates that it may protect post-mitotic neurons through inhibition of the cell cycle. To see if this result was specific to neuronal HT22 cells, SIRT5 was expressed in the NIH/3T3 fibroblast cell line, and here too it induced death (Fig 4.13A). We sought to understand if the death induced in proliferative cells was the consequence of cell cycle inhibition or by some other means. BrdU labeling of HT22 cells showed a drastic inhibition of cell proliferation in cells expressing ectopic SIRT5 (fig 4.13B). These results were separately confirmed by co-immunostaining SIRT5 transfected HT22 cells for Ki67, a nuclear protein active in all parts of the cell cycle except G_0 and a well known marker of cell proliferation (Fig 4.13C).

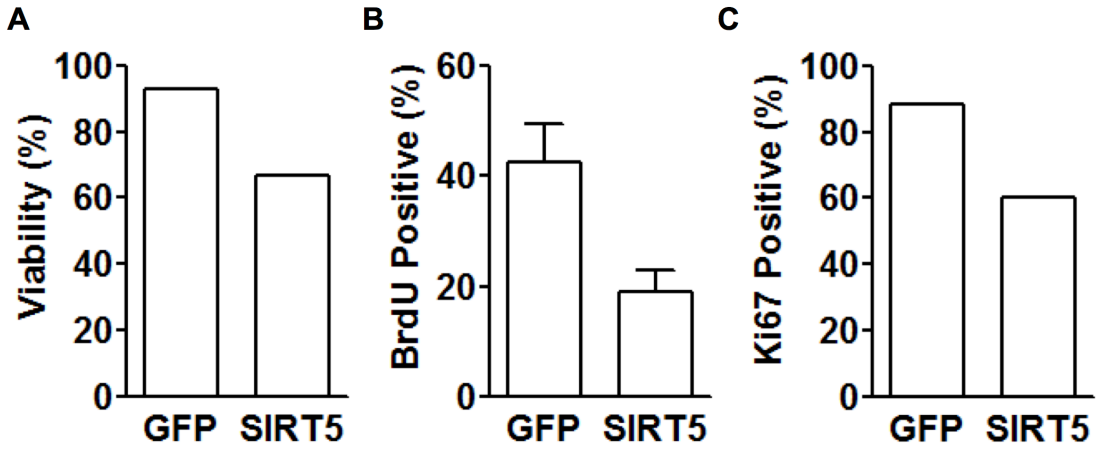


Figure 4.13. SIRT5 induces death in proliferative cells through cell cycle inhibition. *A.* GFP and SIRT5 were transfected into NIH/3T3 cells for 24 h. Viability was quantified by immunocytochemistry with GFP or Flag antibodies and DAPI staining (n=1). *B.* HT22 cells were transfected with either GFP or SIRT5 for 22 h. Cells were then switched to Brdu-containing media (20 μM) for 2 h and then subjected to Brdu labeling. Proliferation was quantified based on both GFP or Flag and BrdU staining (n=2). *C.* HT22 cells were transfected with GFP or SIRT5 for 24 h. Immunocytochemistry was then performed with either a GFP or Flag antibody and co-label with a Ki67 antibody (n=1).

Interestingly, when examining SIRT5 mRNA from a whole brain perspective in a developmental time course of a rat, we find that its expression increases and plateaus from postnatal day 7 (P7) through 12 months of age, indicating an increase in differentiated neurons (Fig 4.14A). At P7, SIRT5 mRNA is relatively ubiquitously expressed across the entire brain (Fig 4.14B).

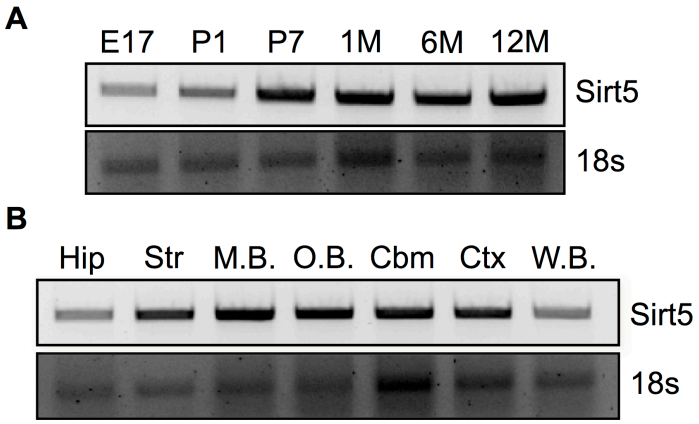


Figure 4.14. SIRT5 mRNA is increased differentiated neurons. *A*. mRNA was extracted from rat whole brain at embryonic day 17 (E17), post natal day 1 (P1) and 7 (P7), one month (1M), six months (6M) and a year (12M) and RT-PCR analysis was performed. 18S serves as a normalization control. *B*. mRNA was extracted from various brain regions from a P7 rat and RT-PCR analysis was performed. Hippocampus (Hip), striatum (Str), mid brain (M.B.), olfactory bulb (O.B.), cerebellum (Cbm), cortex (Ctx), whole brain (W.B.). 18S serves as a normalization control.

While increasing its expression ectopically might provide protection by restoring the decreases of endogenous SIRT5 seen under LK treatment, we asked by what mechanism is ectopic SIRT5 working to keep the neurons healthy and alive. As the regulation of cellular viability is frequently controlled by signaling pathways, we first examined if SIRT5 is a target of known pro-survival molecules by inhibiting them pharmacologically. As figure 4.15A shows, inhibition of MEK/ERK or PI3-K/AKT signaling as well as CamKII had no effect of SIRT5 protection. However, protection was blocked by H89, a PKA specific inhibitor (Fig 4.15B). To further validate PKA involvement, we used an additional inhibitor known as PKI (14-22), which is a

peptide whose sequence is taken from the naturally occurring heat-stable protein kinase inhibitor, PKI (49). The peptide is myristoylated at its N-terminal to increase cell permeability. At two different concentrations PKI (14-22) similarly inhibits SIRT5 protection (Fig 4.15B). Interestingly, nicotinamide and sirtinol, sirtuin specific inhibitors, also had no effect (Fig 4.15C), suggesting that, like SIRT1, SIRT5 is protecting in a deacetylase-independent manner. However, as described earlier SIRT5 has may possess weak deacetylase activity. Further similar to SIRT1, its protective effect against LK-induced death is also blocked by three different classical HDAC inhibitors (Fig 4.15D). While yet to be fully investigated, these results suggest that SIRT5-mediated protection is dependent on the activity of both PKA and a classical HDAC. The HDACi -2 listed in figure 4.15D is the same Class I-specific HDAC inhibitor used above against SIRT1. Since HDAC3 is highly toxic to neurons, it seems unlikely that SIRT5 would require its deacetylase activity. Our lab has shown that HDAC3 toxicity is dependent on activate GSK-3β, a molecule known to induce death in neurons (5). Furthermore, as they work together, HDAC1 toxicity is prevented by inhibiting GSK-3β, and indeed becomes protective against LK-induced death (3). As described above, HDAC1 and 2 can regulate cell cycle progression. Since GSK3β is a downstream target of and inhibited by PKA, and in knowing that an HDAC is important of SIRT5, we investigated if SIRT5 expression altered GSK-3 activity. Interestingly, when expressed in HT22 cells treated with HCA, SIRT5 induced the phosphorylation of GSK3β, a modification that leads to its inhibition (Fig. 4.16). This lends the idea that SIRT5 may protect against LK-induced death by inhibiting aberrant cell cycle induction through cooperation with PKA and a class I HDAC potentially involving inhibition of GSK3β. Indeed, inhibition of GSK3β protects CGNs against LK-induced apoptosis (3, 5). Further, inhibition of GSK3β by

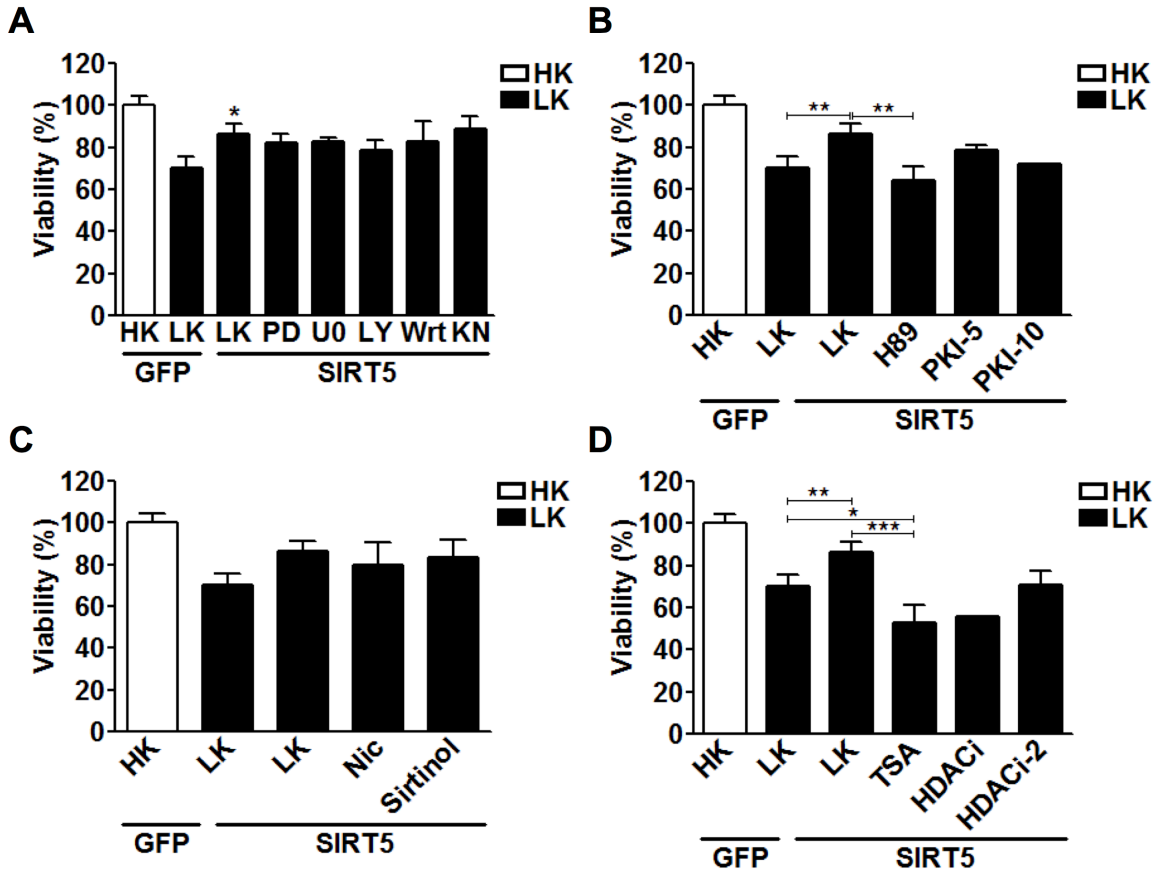


Figure 4.15. SIRT5-mediated protection is blocked by inhibitors targeting PKA and classical HDACs. *A-D.* GFP and SIRT5 were transfected into CGNs for 24 h. Media was then switched to either HK, LK or LK supplemented with the following inhibitors for 24 h: *(A)* PD98059 (PD, 50 μM), U0126 (U0, 10 μM), LY294002 (LY, 10 μM), Wortmannin (Wrt, 100 nM) and KN-62 (KN, 10 μM). *, $p<0.05$ (n=2) *(B)* H89 (3 μM, n=3), PKI (14-22) at 5 μM (PKI-5, n=2) or PKI (14-22) at 10 μM (PKI-10, n=1). **, $p<0.01$. *(C)* Nicotinamide (Nic, 5 mM, n=3) and Sirtinol (100 μM, n=2) and *(D)* TSA (1 μM, n=3), HDACi (50 μM, n=1) and Pimelic Diphenylamide 106 (HDACi-2, 10 μM, n=3). *, $p<0.05$; **, $p<0.01$; ***$p<0.001$. Viability was quantified by immunocytochemistry and DAPI staining with GFP or Flag anitibodies.

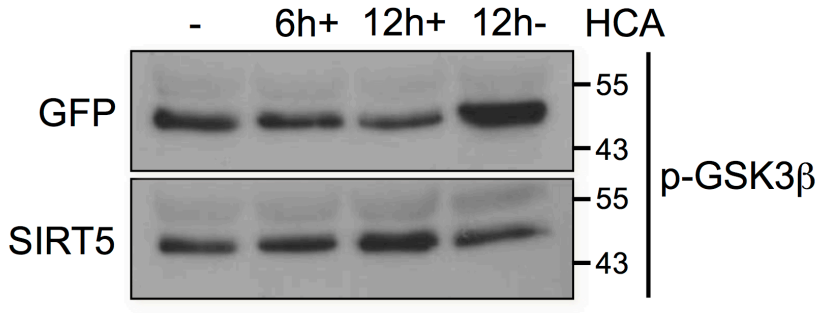


Figure 4.16. SIRT5 expression results in GSK3 phosphorylation after HCA treatment. HT22 cells were transfected with GFP or SIRT5. Cells were then treated with 1mM HCA for either 6 h (6+) or 12 h (12+), left untreated for 12 h (12-) or taken for lysate pre-treatment (-). Following treatment, cells were lysed, subjected to western blotting and probed for phospho-GSK-3(α/β) (Ser21/9).

IGF-1, treatment of which protects CGNs, regulates CGN progenitor proliferation (51). Reduced cerebellar granule cell progenitor proliferation is also seen due to Wee1 stabilization following GSK3β inhibition (52). GSK3β is a well-known regulator of both Wnt and hedgehog (Hh) signaling pathways that are critical for embryonic development and thus mediate expression of cell cycle regulatory genes, most notably cyclin D1 (53). Cyclin D1 expression is also regulated by transcriptionally and protein degradation by GSK3β itself (54). As described in chapter 1, cyclin D1 is a major regulator of the G_1 phase of the cell cycle, as well as the G_1/S transition. Nuclear levels of cyclin D1 have also been found increased in LK-induced CGNs (55) and it is an essential mediator of apoptotic neuronal death (56). Interestingly PKA have been shown to phosphorylate cyclin D1 (57). However, the increase in GSK3β phosphorylation following HCA treatment and SIRT5 expression lends confusing results. As figure 4.16 shows, at 12 h without HCA treatment, GSK3b shows a dramatic increase in phosphorylation with GFP expression compared to 12 h with HCA, however the opposite is true for SIRT5 expression. While SIRT5 inhibits HT22 proliferation and thus induces death, GFP has no effect on cell cycle progression.

Thus the increased phosphorylation of GSK3β with GFP expression, and decrease with SIRT5, at 12 hours without HCA should indicate that this modification is necessary for HT22 proliferation. Further, as phosphorylation is dramatically decreased at 12 with HCA treatment and GFP expression, it appears necessary for HT22 death. However, as SIRT5 increases death in HT22 cells following HCA treatment (11), it would not be expected to show an increase in GSK3β phosphorylation. SIRT5 also fails to protect cortical neurons from HCA-induced cell apoptosis (Fig 4.11). While protection against death by HCA treatment in cortical neurons and HT22 does require CDK inhibition (58), it is possible that SIRT5's ability to regulate neuronal viability is stimulus-specific. Apart from its role in regulating cytochrome *c*, little is known about its role in oxidative stress. However, it has been shown as downregulated following oxidative stress-induced apoptosis in cardiomyocytes (59). HCA treatment can also induce death due to excitotoxicity (60, 61) but SIRT5 has also shown a protective role against this type of death (62, 63). Thus while SIRT5 has revealed itself to be a clear mediated of in the fight against apoptosis, more elucidation is required to fully understand what its roles are.

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VITA

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