Regulation of the Ku70-Bax complex in Cells

By

Manila Hada

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Doctoral Committee:

Associate Professor Roland P.S. Kwok, Chair
Professor Phillip C. Andrews
Associate Professor Jorge A. Iñiguez-Lluhi
Associate Professor Patrick J. O'Brien
Professor Michael D. Uhler

Dedication

To my father, Madhusudan Raj Hada,

And my mother, Bina Hada

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List of Abbreviations

Bak: Bcl-2 Homologous Antagonist Killer

Bax: Bcl-2 associated X protein

Bcl-2: B-Cell Lymphoma-2

Bcl-xL: Bcl long

BH: Bcl-2 Homology

Bid: BH3 Interacting Death domain

Bok: Bcl-2 related Ovarian Killer

CBP: CREB-binding protein

Cdk-2: Cyclin-dependent Kinase 2

CHAPS: 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate

COX IV: Cytochrome c Oxidase Subunit IV

DC4: DABCO Crosslinker containing four carbon atoms in each spacer arm

DISC: Death Inducing Signaling Complex

DMEM: Dulbecco's Modified Eagle's Medium

DNA PKcs: DNA Protein Kinase Catalytic Subunit

DSB: Double Strand Break

ECL: Enhanced Chemiluminescence

FLIP: FLICE-like inhibitory protein

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HDAC6: Histone Deacetylase 6

HEK293: Human Embryonic Kidney -293 cells

HSP 90: Heat Shock Protein-90

Mcl-1: Myeloid Cell Leukemia-1

MEM: Eagle's Minimum Essential Medium

MTT: (3-(4,4 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide)

MOMP: Mitochondrial Outer Membrane Permeabilization

NB: Neuroblastoma

NHEJ: Non Homologous End Joining

NLS: Nuclear Localization Signal

N-Type: Neuronal-type

OMM: Outer Mitochondrial Membrane

PVDF: Polyvinylidene difluoride

SAHA: Suberoyl anilide hydroxamic acid

SAP: SAF-A/B Acinus and PIAS motifs

SCID: Severe combined immunodeficiency syndrome

SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

siRNA: Small interfering RNA

SIRT3: Sirtuin (silence mating type information regulation 2 homolog) 3 (*S. Cerevisiae*)

S-Type: Stromal-Type

t-Bid: truncated-Bid

TNF: Tumor Necrosis Factor

TSA: Trichostatin A

V (D) J: Variable (Diversity) Joining

XRCC4: X-Ray Repair Complementing Defective Repair in Chinese Hamster Cells 4

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Abstract

Using a neuronal cell type (N-type) of neuroblastoma (NB) cells, we have proposed a model in which Ku70, a nuclear DNA repair factor, also regulates a pro-apoptotic protein, Bax, by binding to and blocking Bax-dependent cell death activity in the cytosol. Ku70-Bax binding is regulated by Ku70 acetylation such that when Ku70 is acetylated, Bax dissociates from Ku70, triggering cell death. Some studies have suggested that stoichiometry binding of Bax to Ku70 is critical for this regulation. However, this is not consistent with current literature in which Bax is found to be inactive and monomeric in the cytosol. In this project, I have addressed this issue by determining whether all Bax binds to Ku70 in the NB N-type cells. Furthermore, we have also demonstrated that in NB N-type cells, Ku70 depletion triggers Bax-dependent cell death, suggesting that Ku70 may act as a survival factor. I have also addressed the question whether Ku70 acts as a survival factor in other cell types. Our results showed that in the N-type NB cells only a small fraction of Ku70 binds to a small fraction of Bax; the majority of Bax is monomeric. Interestingly, the majority of Ku70 is in several high molecular weight complexes, and there is no free Ku70 in the cytosol in these cells suggesting that the availability of Ku70 may be another factor that regulates Bax activity. Also, my results show that Ku70 may not be required for survival in some cell types because Ku70 depletion does not affect survival. Furthermore, Ku70 acetylation does not dissociate or activate Bax in these Ku70-depletion insensitive cells suggesting that additional factors may be involved in regulating Ku70-Bax binding.

Chapter 1

Introduction

Apoptosis, programmed cell death, is triggered by Bax activation. One of the factors that regulate Bax activation is Ku70, a DNA repair factor. Previous research has suggested the model that Ku70 binds and inactivates Bax [1]. The binding between Ku70 and Bax is regulated by Ku70 acetylation such that when Ku70 is acetylated, Bax dissociates, triggering cell death. My project focused on understanding the regulation of Ku70-Bax complex in cells.

1.1 What is apoptosis?

Apoptosis is a form of programmed cell death described by Kerr, Wylie and Currie in 1972 [2]. Apoptosis is characterized by distinct morphological changes in which cells undergo shrinkage, and where the cytoplasm of the cells becomes dense [3]. The nucleus undergoes fragmentation and chromatin condensation. One of the unique features of apoptosis is that the plasma membrane remains intact until the late phase [4]. The intracellular content of the apoptotic cells does not spill to the extracellular environment. Thus, unlike in other cell death mechanisms, the inflammatory response from the immune cells does not occur apoptosis [2].

Apoptosis can occur via either intrinsic or extrinsic pathways (fig.1.1). The extrinsic apoptotic pathway is activated by death receptor ligands, such as tumor necrosis factors (TNF) and Fas. These ligands bind to the death receptor and form a large death inducing signaling complex (DISC). DISC complex activates the protease caspase 8, which in turn activates the

downstream executioner caspases 3, 6, or 7 [5]. Activated caspase 8 will also truncate Bid (BH3 only protein) proteins forming tBid [6-8] (the BH3 domain proteins will be described in detail below). tBid binds to the mitochondrial membrane and undergoes a conformational change. This activated tBid will in turn activate Bax (described below) [9-11].

The intrinsic apoptotic pathway can be triggered by stimuli like DNA damage, reactive oxygen species, and the unfolded proteins response (fig 1.1) [12, 13]. This pathway is strictly regulated by the members of the Bcl-2 family of proteins [14]. This family of proteins is characterized by the presence of a Bcl-2 homology domain (BH-domain) (fig. 1.2), but even though some family members of Bcl-2 proteins have conserved sequences and exhibited similar structures, they may have opposite activities [15, 16]. Pro-survival proteins [Bcl-2, Bcl-xl (long), and Mcl-1 (Myeloid Cell Leukemia-1)] contain four BH domains. They are usually embedded in the outer mitochondrial membrane (OMM), cytosol, and endoplasmic reticulum. These proteins preserve the outer mitochondrial membrane [17, 18]. Pro-apoptotic proteins, Bax (Bcl-2 associated X), Bak (Bcl-2 homologous antagonist killer) or BOK (Bcl-2 related ovarian killer), consist of three BH domains. Both Bax and Bak are important in triggering apoptosis. However, the function of Bok is still unclear. The Bcl-2 family also contains a subset of proteins that possess only BH3 domains (Bad, Bik, Bid, Bmf, Hrk, Bim, Noxa, Puma). These BH3-only proteins can directly activate the pro-apoptotic proteins (Bax and Bak) or they may interact with the pro-survival proteins and inhibit their actions [19-23].

The balance between the pro-apoptotic and pro-survival proteins of the Bcl-2 (B-cell lymphoma) family of proteins controls the mitochondrial apoptosis pathway by regulating the activity of Bax, a cytosolic pro-apoptotic protein [15, 24]. When Bax is activated, it forms a pore in the outer mitochondrial membrane (OMM) and releases the apoptotic content, such as cytochrome c, SMAC/DIABLO and endonuclease G of mitochondria, to the cytosol. The

formation of the pore in the OMM is usually considered the “point of no return” in the apoptosis process [13, 25-27]. The changes of the mitochondrial membrane potential results in releasing the mitochondrial content including cytochrome c [28]. The released cytochrome c binds to APAF1 and pro-caspase 9 forming complexes known as apoptosomes. The binding of pro-caspase 9 to the apoptosome complex results in the cleavage of pro-caspase 9 that becomes activated. The activated caspase 9 in turn activates downstream executioner caspases resulting in apoptosis [29].

1.2 Bax

Bax belongs to the Bcl2 family of proteins containing 3 BH domains. Bax has multiple isoforms (alpha, beta, gamma, delta, epsilon, zeta, psi, sigma) identified at the mRNA level. Whether all cells express all these Bax isoform proteins is not clear. The only well-characterized isoform of Bax is Bax alpha [30, 31], containing 192 amino acids (21kD). The full length of the alpha Bax protein consists of 9 α helixes. The last helix 9 at the carboxy (C-) terminus of Bax serves as a transmembrane domain, targeting Bax to mitochondria [32-34]. Helix 9 is embedded in a hydrophobic groove interacting with the amino (N-) terminus of the protein maintaining Bax as a monomer and inactive form [35]. The N-terminus of Bax consists of the N-segment (residues 1-15) and helix 1. N-segment is unstructured and exposed to solvent and interacts with helix 9. When residues 1-20 are truncated, the mitochondria targeting sequence at helix 9 is exposed targeting Bax to mitochondria [36, 37]. Thus the N-segment is responsible for retaining Bax in an inactive form in the cytosol. Following apoptotic stimuli (by binding to the Bcl-2 BH3 only proteins. For example) the N-segment undergoes conformational changes exposing helix 1 and releasing helix 9. In an effort to detect activated Bax, an antibody (6A7) was raised

against residues 1-19 of Bax, which is exposed when Bax is activated. Thus, this antibody has been used as a marker for Bax activation [38].

While studies have shown that Bax is inactive and monomeric, how Bax is activated is still controversial [25, 39]. Some suggested that Bax is activated by the direct interaction with the BH3-only proteins. However this interaction is yet to be established in cells [40-42]. One reason for not detecting the interaction between BH3-only proteins and Bax is that it may be a 'hit and run' mechanism forming a transient interaction between BH3-only proteins and Bax [9, 20]. The conformational change in Bax after interaction with BH3-only proteins may release BH3-only proteins immediately [16]. BH3-only proteins also activate Bax by directly binding to pro-survival proteins and inhibiting their action.

In addition to binding to the Bcl-2 family of proteins, Bax also binds to other non Bcl-2 family proteins, such as Humanin, 14-3-3, and Ku70 [1, 43, 44]. Humanin and 14-3-3 have been shown to bind Bax when they were overexpressed, but it is not known whether they regulate Bax activity. The only well studied non-Bcl2 protein that binds to Bax is Ku70 [1, 45, 46]. Our lab focuses on the regulation of binding between Ku70 and Bax.

Bax activation is also regulated by post-translational modifications, such as phosphorylation and ubiquitination [47, 48]. Akt or protein kinase B, a serine-threonine kinase, phosphorylates Bax at S184 in helix 9 [49], inhibiting Bax translocation to mitochondria [50]. Conversely, Bax dephosphorylation at S184 by PP2A activates Bax [51]. However, phosphorylation of Bax at S163 by GSK3β in neuronal cells activates Bax [47].

1.3 Biochemistry and function of Ku protein

Ku70 was originally identified as an auto-antigen in the sera of a patient with autoimmune disease [52, 53], and subsequently it was characterized as a double strand break (DSB) DNA repair factor [54]. In the nucleus, Ku70 forms a heterodimer with Ku80, and this dimer binds to DNA at the site of double strand break to initiate the first step of the Non Homologous End Joining (NHEJ) DNA repair process [55-57]. Ku70 and Ku80 have been implicated to form an obligate heterodimer i.e. each subunit is unstable in the absence of another [58-60]. Though their primary sequences vary, their secondary structures are similar [61, 62]. The full length Ku70 and Ku80 heterodimer structure has been elucidated with or without DNA in the complex by single particle electron microscopy [63, 64]. Each Ku protein has 3 domains: an amino (N) terminal α/β domain, a central β-barrel domain and helical carboxyl (C) terminal domain. The α/β domain of Ku70 and Ku80 binds to other proteins involved in DNA repair. Ku70 and Ku80 dimerize through the central domain and form a ring that binds to DSB DNA [65]. The Ku70 C-terminal domain is flexible and is followed by a structured SAP domain (named after SAF-A/B, Acinus, and PIAS motifs). SAP domain increases the overall binding affinity to DNA [66-68].

In addition to its role in DNA DSB repair, Ku70/Ku80 is implicated in telomere function [69]. The exact mechanism of Ku association with the telomere is still not clear. It is not known whether Ku70 binds directly or by protein-protein interaction at the telomeres. Cells from Ku proteins deficient mice exhibit fused telomere and chromosomal aberrations [70, 71]. Studies have shown that Ku proteins promote telomere addition by recruiting telomerase [72, 73]. The presence of Ku proteins at the telomere ends also protects telomeres by inhibiting nucleolytic degradation [74, 75].

Ku proteins have also been implicated in different diseases. Ku70-Ku80 deficiency results in various immune abnormalities. Ku70/Ku80 knockout mice have abnormal B-cell and T-cell development, and lymphocyte differentiation [76, 77]. The defect in the Variable (Diversity) Joining [V-(D)-J] recombination leads to severe combined immunodeficiency syndrome (SCID) [78]. Increased aging and senescence are observed in Ku deficient mice. This might be explained by the role of Ku proteins in maintaining telomere length. The absence of Ku proteins lead to shortened telomeres length contributing to accelerated aging [79, 80].

As Ku protein plays an important role in maintaining chromosomal integrity and cell survival, its role in various cancer models has been studied [81]. Ku70 expression in tumors is associated with unfavorable responses to conventional chemotherapy and radiation treatment. Ku70 expression in cervical carcinoma is inversely correlated with radiation sensitivity and patient survival. Thus ectopic overexpression of Ku70 leads to resistance to agents inducing DNA break or Bax activation [58, 82-84].

1.4 Regulation of the Ku70-Bax complex in cells

While Ku70 is known as a DNA repair factor in the nucleus, it has also been found in the cytosol [45, 85, 86]. While looking for Bax inhibiting factors by yeast-two hybrid assay, Sawada et. al. found that Ku70 was one of the factors that interact with Bax [1]. The Bax binding domain of Ku70 has been mapped to the C-terminal 578-609 of Ku70. A Bax-binding peptide has been designed that corresponds to Ku70's Bax-binding domain sequence and this peptide binds to Bax and inhibits Bax mediated apoptosis [87]. The binding between Ku70-Bax is regulated by acetylation by the CREB-binding protein, CBP [85, 88, 89]. There are at least eight known acetylated lysines within the Ku70 molecule: K282, K331, K338, K539, K542, K544, K553 and K556 [89]. Two of these lysines K539 and K542, are located within the linker region of Ku70.

Acetylation of these two lysines causes a conformational change at the Bax-binding region of Ku70, allowing Bax to dissociate from Ku70. The function of other acetylable lysines within Ku70 is still not clear. For example, K282 and K317 are found within the DNA binding domain of Ku70 [90]. One study has suggested that acetylation of these residues may reduce Ku70 binding affinity to DNA [91]. Furthermore, K539, K542, K544, K533 and K556 are found in the nuclear localization signal (NLS) of Ku70. Acetylation of these lysine residues may regulate the subcellular localization of Ku70 [92]. Whether, acetylation of these lysines affects Ku70 localization is not clear.

Ku70 acetylation is also regulated by two deacetylases: SIRT3 and HDAC6 (Histone Deacetylase 6) [45, 93-95]. However, SIRT3 is a mitochondrial protein, and it is unclear how a mitochondrial deacetylase deacetylates a cytosolic protein. Studies from our laboratory as well as from other laboratories have shown that HDAC6 deacetylates Ku70. HDAC6 is a class II b HDAC found in the cytosol. HDAC6 has been implicated in many cellular functions including tubulin stabilization, cell motility, and the regulation of the binding between HSP90 (Heat Shock Protein 90) and its essential co-chaperone p23 [96]. We have shown that HDAC6 forms a complex with Ku70 and Bax. Depletion of HDAC6 using HDAC6 specific siRNA increases Ku70 acetylation and results in cell death [94]. Furthermore, treatment with class I HDAC inhibitors, such as TSA and SAHA, or a HDAC6 specific inhibitor, such as tubacin, induces Ku70 acetylation and results in cell death. These results suggest that regulation of Ku70 acetylation is a factor in controlling Bax activity.

In addition to Bax binding, cytosolic Ku70 has been shown to interact with other cytosolic factors, such as p18-cyclin E, FLIP, and Caveolin. Cyclin E/ Cdk2 (Cyclin-dependent kinase 2) complex plays an important role in cell cycle transition and DNA replication. Caspase mediated cleavage of cyclin E forms p18-cyclin E, which binds to cytosolic Ku70 and induces Bax

dissociation leading to apoptosis. However, the function of p18-cyclin E in regulating Bax activity is not clear [97]. Another pro-survival protein, FLIP (FLICE-like inhibitory protein), like Bax, also binds to Ku70 in an acetylation dependent manner. FLIP binds to the Ku80-binding domain of Ku70. When Ku70 is acetylated at the same two lysines, K539 and K542, Bax will dissociate from FLIP, triggering FLIP poly-ubiquitination and degradation [45]. Whether FLIP and Bax bind to Ku70 simultaneously and dissociate from Ku70 when Ku70 is acetylated at K539 and K542 is not clear (as discussed in Chapter 3).

1.5 Our Model and Questions

In the past several years, using SH-SY5Y cells, a neuronal type (N-type) neuroblastoma (NB) cells, we have been investigating how Ku70 regulates Bax activities in cells. NB is the most common extracranial solid pediatric cancer [98, 99]. The tumor arises from the sympathoadrenal neural crest stem cells [100]. NB tumors are highly heterogeneous, being principally comprised of tumor cells that are classified as either neuronal (N) type or stromal (S) type cells [101]. S-type cells are noninvasive and adherent cell types while N-type cells are aggressive expressing neuronal markers such as tyrosine hydroxylase and dopamine-β-hydroxylase. N-type cells express high level of the N-myc proteins and are more commonly isolated from high-risk tumor explants. Thus, N-type cells are used to model a more aggressive and highly transformed NB cell type [101].

In these SH-SY5Y cells, we have shown that Bax activation is regulated by binding to Ku70 [102] (fig. 1.3). Ku70 binding to Bax inhibits Bax's pro-apoptotic activity. We and others have demonstrated that Ku70-Bax binding is regulated by acetylation of Ku70 such that when Ku70 is acetylated by the CBP, Bax dissociates from Ku70 [45]. The dissociated Bax enters mitochondria releasing cytochrome C triggering apoptosis. Ku70 acetylation is also regulated by

HDAC6 such that HDAC6 binds and deacetylates cytosolic Ku70. Inhibition of HDAC6, either by using class I and II HDAC inhibitors, such as SAHA and TSA, or by HDAC6-specific inhibitors, tubacin, or by depleting HDAC6 using siRNA, increases cytoplasmic Ku70 acetylation and results in Bax dissociation [94]. This model, however, assumes that all cytosolic Bax binds to and is regulated by Ku70. However, this model is inconsistent with a long-held model that Bax is monomeric and is found to be inactive in the cytosol [39]. Previously studies have shown that in unstimulated cells, using anti-Bax antibodies (6A7) that recognize activated Bax, a small amount of Bax was immunoprecipitated. However, following the treatment of Bax-activating compounds, like staurosporine, 6A7 antibody was able to pull down more Bax [45]. Furthermore, when Bax is analyzed by gel filtration chromatography, it was revealed that Bax is found in fractions at molecular weight corresponding to 29 kD proteins [103]. Here in this thesis, I have addressed two questions: The first question is whether all Bax binds to Ku70. Our results show that only a small fraction of Bax binds to a small fraction of cytosolic Ku70. The majority of Bax is monomeric. However, the majority of cytosolic Ku70 is in complex with other factors forming several high molecular weight complexes. There is no free or monomeric Ku70 found in the cytosol. These results suggest that other factors may also regulate Ku70-Bax binding by restricting the availability of Ku70 to bind Bax (fig. 1.4).

In SH-SY5Y cells, we have shown that Ku70 depletion triggers Bax-dependent cell death, suggesting that Ku70 may act as a survival factor in these cells. The second question I have addressed in this thesis is whether the Ku70 acting as a survivor model in SH-SY5Y cells is also applicable to other cell types. I have provided evidence demonstrating that, in multiple cell types (SHEP-1, ES2, A2780, and HEK-293T cells), depletion of Ku70 does not affect cell survival. Interestingly, these cells are also not sensitive to HDAC inhibitor killing compared to that of N-type NB cells. Moreover, in these cells, while HDAC inhibitor treatment increases cytosolic Ku70 acetylation, Bax is not activated nor it dissociates from Ku70. These results

suggest that there may be another mechanism that regulates Ku70-Bax formation and Bax activation in these cells (fig. 1.4). Collectively, our results suggest that there may be at least two cell types in terms of Ku70 regulating Bax function: one is Ku70-depletion sensitive cells in which at the basal level Ku70 acts as a survival factor (like that in SH-SY5Y cells); the second cell type is Ku70-depletion insensitive cell type in which Ku70 is not required for survival, but Ku70 inhibits Bax activity when Bax is activated.

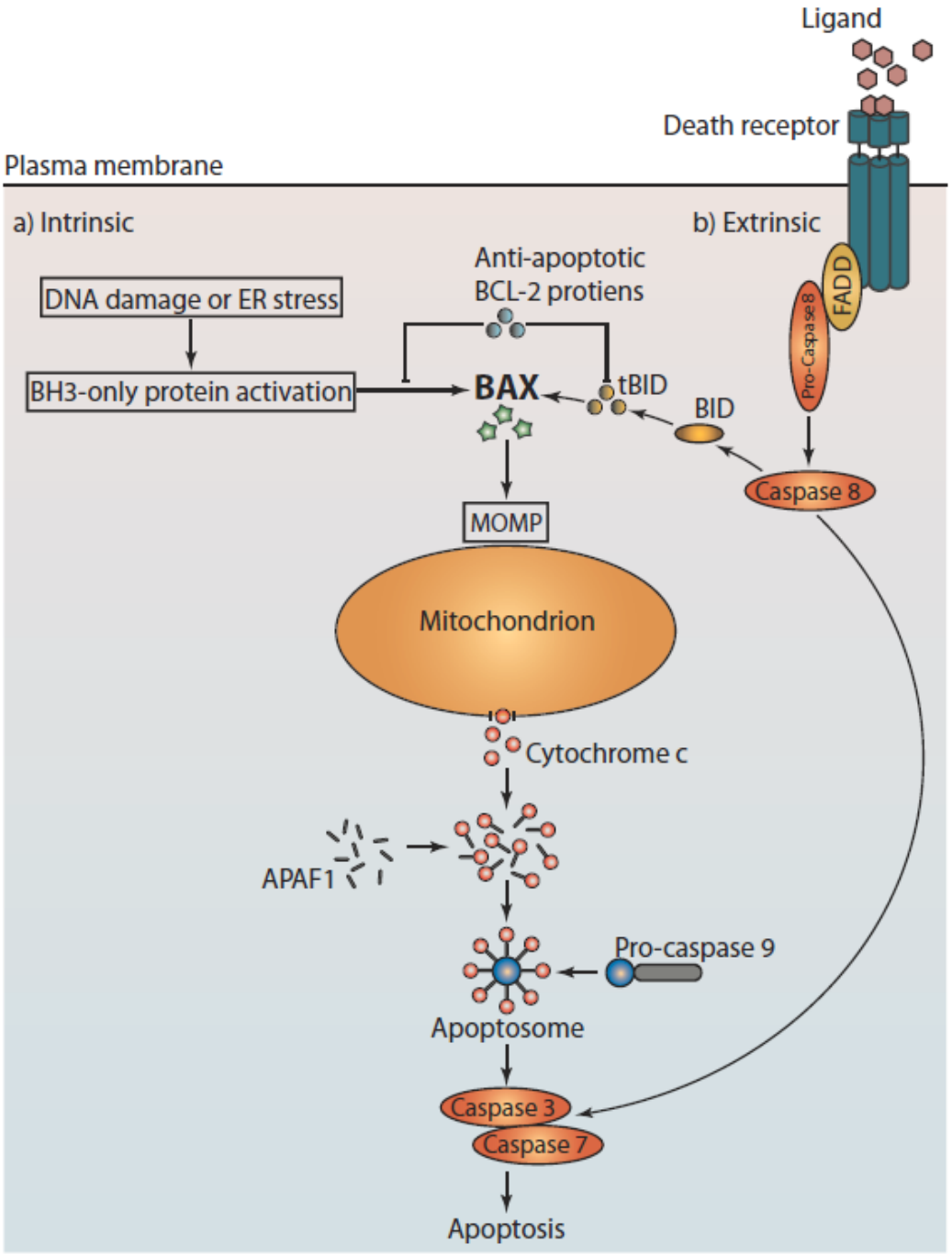

Figure 1.1: Schematic of Intrinsic and Extrinsic pathways of apoptosis In the intrinsic pathway, Bax is activated by internal stimuli, like DNA damage. Activated Bax translocates to the mitochondria, causing the release of cytochrome c, which triggers the formation of apoptosome, activating pro-caspase 9, resulted in apoptosis. The extrinsic pathway is triggered by external stimuli, like the tumor necrosis factor, which binds to the dead receptor, activating caspase 8. Activated caspase 8 has two possible routes to induce apoptosis. One is to directly activate downstream caspases, like caspase 3, or it truncates a Bcl2 protein BID, forming tBID, which in turn activates Bax.

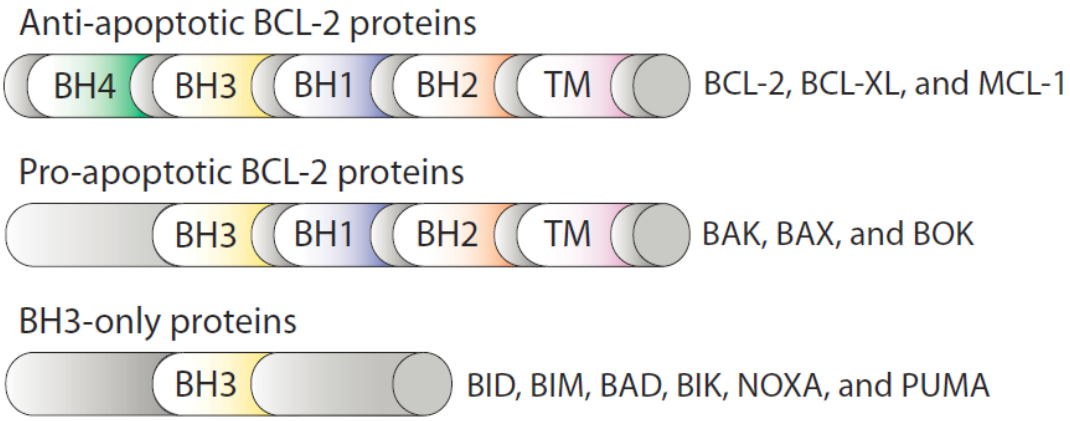

Figure 1.2: B cell lymphoma 2 (BCL-2) family of proteins The Bcl2 family of proteins can be classified into three groups depending on the number of the Bcl2 homology (BH) domains. Proteins containing 4 BH domains, such as Bcl2 and Bcl-XL, are mainly anti-apoptotic proteins. Bax, Bax, and Bok contain 3 BH domains and are mainly pro-apoptotic. BH3-only proteins are mainly pro-apoptotic and either bind to the pro-apoptotic proteins directly or by inhibiting the function of anti-apoptotic proteins.

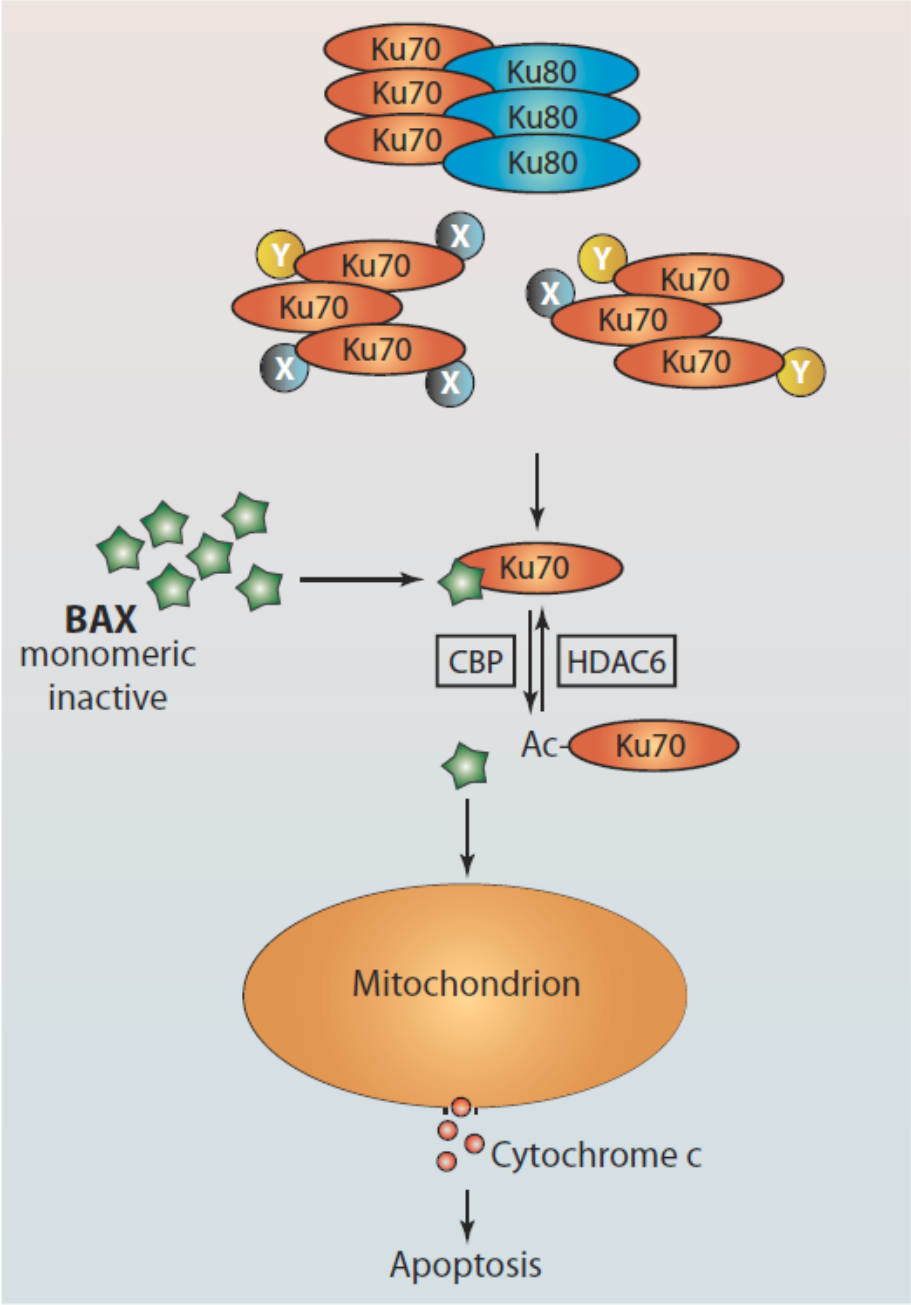

Figure 1.3: A model for the regulation of Ku70-Bax complex in cells Our model indicates that Bax is monomeric and only a small fraction of Bax binds to a small fraction of Ku70. The majority of Ku70 forms complexes with other factors, including Ku80. There is no free Ku70 in the cytosol. This model suggests that the availability of Ku70 to bind to additional activated Bax may be a factor in regulating Bax's cell death activity.

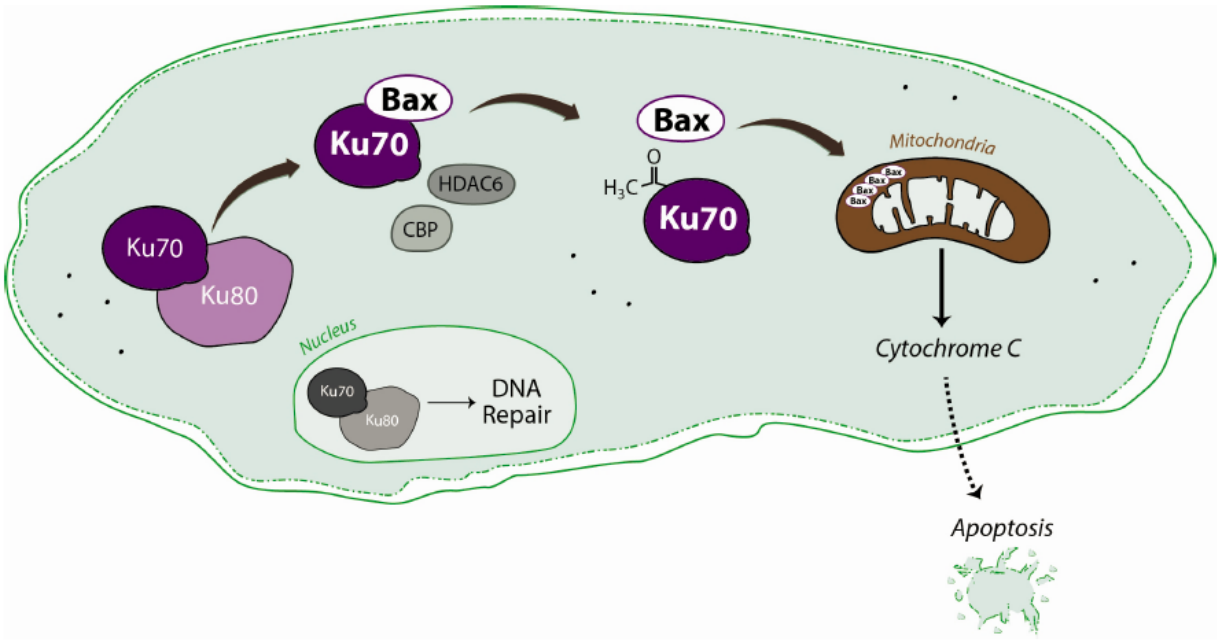

Figure 1.4: Schematic representation of Ku70-Bax regulation by cytosolic Ku70 complexes Using N-type NB cells as a model, we have shown that Ku70 binds to Bax in an acetylation dependent manner. When Ku70 is acetylated, Bax dissociates from Ku70, translocating into mitochondria, triggering the release of cytochrome C, resulted in apoptosis. Depletion of Ku70 in these cells also triggers Bax-dependent cell death. This model suggests that all Bax in cells must bind to Ku70 [102]

Chapter 2

Materials and Methods

Cell Culture

HEK-293T, two ovarian cancer cell lines (ES2 and A2780) and human NB cell lines SH-EP1, SH-SY5Y, SH-EP1, GOTO, KCN-69n and LA1-5s were cultured in modified Eagle's minimum essential medium (MEM) supplemented with sodium pyruvate and 10% fetal bovine serum, and maintained at 37^{o}C in a humidified 5% CO_2 incubator. The human male foreskin fibroblasts were cultured in DMEM (Dulbecco's Modified Eagle's Medium) with 10% fetal bovine serum.

Cell Viability Assays

Cell viability was determined by either MTT [(3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide)] or trypan blue exclusion assay. For the MTT assay, 96 well plates were used. N-type SH-SY5Y, S-type SHEP1 human NB cell lines and human male foreskin fibroblasts were treated with varying concentrations of SAHA (Suberoyl anilide hydroxamic acid). The human ovarian cancer cell lines A2780, ES-2 were treated with varying concentrations of TSA (Trichostatin A). The viability of the cell lines was determined after 24 and 48 hours post treatment by MTT assay as previously described [104]. All experiments were carried out three times with triplicates in each experiment and the average values and the standard deviations were calculated.

siRNA mediated silencing and over expression of Flag-Ku70 and Flag-Ku80

For Ku70 knock down using siRNA (small interfering RNA) experiments, cell lines were plated at a density of ~$2x10^6$ cells per 10 cm plate 24 hours before transfection. The following day the cells were transfected either with smart pool Ku70 siRNA (silencing RNA) or the scrambled non-targeting siRNA (Dharmacon Inc.) using nucleofector kit V (Amaxa) as per the manufacturer's instruction. Mock transfection as well as the non-targeting siRNA transfection served as controls. The level of Ku70 was measured 72 hours after transfection by immunoblot analysis using Ku70 antibodies. Either GAPDH or β-tubulin was used as a loading control. The viability of cells after knockdown was measured by counting cells using trypan blue exclusion analysis.

The cell line SH-SY5Y was transfected with pCMV-2B-Flag-Ku70, pCMV2B-Flag-Ku80 or pCMV-2B vector alone using Lipofectamine 3000. Twenty-four hours after transfection, the cells are re-transfected with the expression vectors again. Twenty-four hours after the second transfection, cells are plated into 96 well plates. The rest of the cells are replated on tissue culture plates. One day later, the cells were treated with various doses of SAHA. Forty-eight hours after SAHA treatment, cell viability was determined by MTT assay. The level of expression of transfected Ku70 or Ku80 were determined by immunoblotting experiments using anti-Flag antibodies, anti-Ku70 antibody, and Ku80 antibody. All experiments were carried out three times with triplicates in each experiment and the average values and the standard deviations were calculated.

Western blot Analysis

The proteins were separated by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis), transferred to PVDF (Polyvinylidene Difluoride) membrane and then the blot was blocked using 5% milk. After blocking, blots were probed with different antibodies specific for different proteins. The following antibodies were used for western blot analyses: Ku70 (N3H10) from Santa Cruz; Ku80 (#2753), Bax (#2772S), anti-Bax antibody [6A7] (ab5714), COX IV (Cytochrome c Oxidase Subunit IV) (#4844), and acetylated lysine (Ac-K-103) from Cell Signaling; GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) (6C5) from Millipore and β-tubulin from Upstate. Flag antibody (F3165) was from Sigma-Aldrich. The presence of protein was visualized by using Lumigen ECL (Enhanced Chemiluminescence) Plus PS-3 and Carestream Kodak BioMax Films.

Immunoprecipitation

Co-immunoprecipitations of Ku70, Ku80, and Bax were performed in CHAPS buffer according to the protocol described by Sawada et al. [105] with some modifications. Cells were lysed using lysis buffer (20 mM HEPES, pH 7.5, 120 mM NaCl, and 1% CHAPS) on ice for 30 minutes. The extraction solution was spun twice 3000 rpm for 10 min. The protein concentration was determined by Bradford protein assay. One milligram of protein in 500 μl lysis buffer was used for immunoprecipitation.

Subcellular Fractionation: Cytosolic and Mitochondrial fraction

Cells were grown in 10cm plates to 80% confluency. The cells were washed twice with PBS, and the cells were suspended in the mitochondrial isolation buffer (5 mM HEPES pH 7.5, 210 mM Mannitol, 70 mM Sucrose, 1 mM EDTA) at 1 million cells /100 ml of buffer. The cells were swollen on ice for 30 minutes, and were dounced in a dounce homogenizer for 50 strokes. The lysed cells were pelleted at 1500 rpm for 5 minutes at 4°C. The supernatant was recentrifuged at 3000 rpm for 10 minutes. The remaining supernatant which contained cytosolic and mitochondrial fractions was recentrifuged at 10,000 g for 20 minutes at 4°C. The supernatant was collected as the cytosolic fraction and the pellet was collected as mitochondrial fraction. The mitochondrial pellet was washed once with the mitochondrial isolation buffer. Equal amounts of cytosolic and mitochondrial protein were separated by SDS-PAGE. The purity of mitochondrial fraction or cytosolic fractions was determined by immunoblotting with COXIV and β-tubulin antibodies, respectively.

DC4 Crosslinking

DC4 crosslinker was used in our cross-linking analyses [106]. Crosslinking was carried out in a buffer containing 100 mM HEPES, pH 7.5, 150 mM NaCl. Each cross-linking reaction contained 10-15 μg of cytosolic or nuclear proteins with 0, 0.1, 0.2, 0.4, 0.8, or 1.6 mM of DC4 per each sample. The crosslinking reaction was carried on ice for 30 minutes. The reaction was stopped by SDS-loading buffer. Crosslinked samples were separated by 10% SDS-PAGE, and the blot was probed with Ku70, Ku80, or Bax antibodies.

Gel filtration

Gel filtration chromatography was carried out using a Bio-Rad Biologic DuoFlow system on a Superdex 200 HR 10/30 column at a flow rate of 0.5 ml per min. One milligram of cytosolic protein extract was injected into the column. The running buffer was the same as the extraction buffer containing 20 mM HEPES, pH 7.5, 120 mM NaCl and 1% CHAPS. Half a milliliter fractions were collected per fraction. Twenty microliters of each fraction was separated by SDS-PAGE, and the blot was probed with Ku70, Ku80, or Bax antibodies.

Chapter 3

Interaction between Ku70 and Bax

3.1 Introduction

Ku70 was originally described as an auto-antigen [107]. Later, it was characterized as a DNA-binding factor in NHEJ DSB DNA repair [108]. In a yeast two-hybrid study, Ku70 was found to be one of the factors that bind and inhibit Bax activity [109]. Studies from our lab and others demonstrated that Ku70-Bax binding is important in regulating Bax-dependent cell death [110, 111]. The current model is that Ku70-Bax binding is regulated by Ku70 acetylation such that when Ku70 is acetylated by CBP, Bax dissociates from Ku70 [112]. On the other hand, HDAC6 binds and deacetylates cytosolic Ku70 such that inhibition of HDAC6 by HDAC6 inhibitors or by depleting HDAC6 increases cytosolic Ku70 acetylation resulted in Bax dissociation. The dissociated Bax then enters mitochondria, triggering cytochrome c release, resulting in cell death. One notion of this model is that it is assumed that all Bax interacts with Ku70. However, it is known that Bax is found as inactive monomers in the cytosol. This model is supported by studies showing that in unstimulated cells, anti-Bax antibodies (6A7) that recognize activated Bax can only immunoprecipitate a small amount of Bax. In contrast, 6A7 precipitates a larger amount of Bax when the cells are stimulated by Bax-activating compound like staurosporine [1]. Furthermore, when Bax is analyzed using gel filtration chromatography, it reveals that Bax is found in fractions at molecular weight corresponding to 29 kD proteins suggesting that Bax is monomeric in cells [103]. In this project, we determined the fraction of Bax that binds Ku70, and the fraction of Ku70 that binds Bax.

3.2 Results

Cytosolic Ku70 forms high molecular weight complexes while Bax is found to be monomeric

Gel filtration chromatography reveals that the majority of the cytosolic Ku70 was found in fractions corresponding to high molecular weight complex or complexes, and that Bax was found in a lower molecular weight complex (fig. 3.1) corresponding to 29 kD, as previously described [103]. There was a very little overlap between Ku70 and Bax. These results indicate that not all cytosolic Ku70 interacts with all Bax in the cytosol, there is only a small fraction of each found in the same fractions in the gel filtration chromatograph. Interestingly, the pattern of Ku80 in the gel filtration chromatography was different from that of the pattern of Ku70. The peak of Ku80 was found at 200 kD mark, and it was over lapped with fractions containing Bax.

To investigate whether Ku70 is found in one high molecular weight complex as the results in fig. 3.1 suggested or it is found in many different distinct complexes, we conducted a cross-linking experiment using a cross-linker, DC4, provided by Dr. Phillip Andrews [106]. We cross-linked cytosolic extracts and nuclear extracts of SH-SY5Y cells with various concentrations of DC4 cross-linker as shown in fig. 3.2. Cytosolic Ku70 was found in at least three high molecular weight complexes while nuclear Ku70 was found in only two high molecular weight complexes. One of the Ku70 containing complexes, in the cytosol or in the nucleus, co-migrates with a Ku80 positive cross-linked complex suggesting that they constitute a Ku70-Ku80 complex. However, unlike Ku70 in the cytosol forming several high molecular weight complexes, Ku80 seemed to form only one high molecular weight complex in addition to the complex that was also positive for Ku70. But in the nucleus, all Ku80 seemed to form only one complex that co-migrates with a Ku70 cross-linked complex. Most importantly, however,

there was no monomeric Ku70 or Ku80 in the cytosol and in the nucleus as monomeric Ku70 and Ku80 disappeared in the SDS-PAGE immunoblot when increasing amount of DC4 was used. The cross-linking results of Bax are also consistent with the model that only a very small fraction of Bax is complexed with Ku70 as increasing cross-linking did not seem to reveal Bax-Ku70 complexes due to the sensitivity of the western blot analyses.

Ku80 mainly forms a complex with Ku70 in cells

Ku80, when analyzed by gel filtration chromatography, was found in fractions closer to that containing Bax (fig.3.1), and Ku80 was only overlapped with Ku70 in a few fractions. These results suggest that Bax may interact with Ku80 in cells. However, our immunoprecipitation results did not support this hypothesis, at least in SH-SY5Y cells (fig 3.3). We have shown that, in SH-SY5Y cells, Bax was immunoprecipitated using anti-Ku70 antibody, however, SAHA treatment disrupted this interaction, as we have shown previously [113]. Importantly, immunoprecipitation of Bax, with or without SAHA (HDAC Inhibitor) treatment did not precipitate Ku80. These results suggest that despite the results observed in gel filtration chromatography that fractions of Ku80 were closer to fractions containing Bax, these two proteins may not bind to each other in cells.

We next asked whether Ku70 and Ku80 bind to each other in the cytosol. We immunoprecipitated Ku70 or Ku80 in the cytosolic extracts of SH-SY5Y cells with or without SAHA treatment. We found that Ku70 or Ku80 immunoprecipitated each other with or without SAHA treatment (fig 3.4). Interestingly, both anti-Ku70 antibodies or anti-Ku80 antibodies did not precipitate Bax with or without SAHA treatment, suggesting that the Bax binding to Ku70 (or may be Ku80) may block the epitope of anti-Ku70 antibodies binding domains resulted in failure

in precipitating Bax. It may also be due to the possibility that fraction of Bax that interacts with Ku70 is small.

3.3 Discussion

The main focus of this part of the project is to solve a long unanswered question regarding how Ku70 regulates Bax activity. When this model was established in 2003-2004 [1], despite the overwhelming evidence showing that Bax is an inactive monomer [28], this issue was not discussed. The model assumed that all Bax in the cytosol is regulated by Ku70. While we and others have shown that cytosolic Ku70 binds Bax [45, 91, 114], the stoichiometry of the binding between Ku70 and Bax has never been established.

Why is it important to know the stoichiometry of the binding between Ku70 and Bax? It is because it will distinguish various different competing models of how Ku70 regulates Bax function. The first model is that if all cytosolic Ku70 binds to Bax and all Bax binds to Ku70, it will suggest that the only function of cytosolic Ku70 is to regulate Bax. This model also suggests that the major regulator of Bax function is Ku70 because Ku70 needs to dissociate from Bax in order for Bax to be activated. The second model, which is similar to the first model, is that only a portion of cytosolic Ku70 binds to all Bax in the cell. The remaining Ku70 is either complexed with other proteins, such as Ku80 or is monomeric. This model, similar to the first model, also suggests that Ku70 is the major regulator of Bax function because Ku70 is required to dissociate from Bax in order for Bax to be activated. The second model is also consistent with findings reported in the literature that Ku70 associates with other factors in the cytosol [46, 95, 97]. However, the first and the second models are inconsistent with the previous finding that Bax is an inactive monomer found in the cytosol.

The third model, which is supported by our results shown in this chapter, suggests that only a small fraction of Ku70 and Bax bind to each other. The majority of Bax is still monomeric. Ku70 forms several distinguishable high molecular weight complexes with other proteins. Furthermore, there is no monomeric Ku70 in the cytosol.

These results support a model in which Ku70 acts as a survival factor to protect the cells from dying of Bax-dependent apoptosis. How could a small amount of cytosolic Ku70 protect the cell from dying when there is a large amount of Bax? We believe that cells throughout life constantly receive stimuli, including dead signals that induce cell death. Some of the dead stimuli may lead to apoptosis while some of the small stimuli may only activate a few Bax molecules. As a survival mechanism, cells do not die after receiving weak signals that activate small amount of Bax. To survive these aberrant Bax-activation signals, cells developed a mechanism to block these signals. We believe that Ku70 may act as a survival factor, blocking the small amount of Bax that is being activated by weak cell dead signals.

This model suggests because there is only a small amount of Ku70 that binds to a small amount of Bax, and additional Ku70 may be needed when more Bax is activated. However, where does the additional Ku70 come from if there is no free Ku70, and all remaining Ku70 that does not bind Bax is in complex with other factors? This model suggests another level of regulation of Ku70-Bax binding in which Ku70 has to be released from other complexes, or Ku70 is available from other Ku70-complexes that can bind activated Bax. As discussed above that Ku70 is known to bind to several factors in the cytosol [45, 46, 97]. One of these binding proteins is FLIP, which is an anti-apoptotic protein blocking caspase 8 activation by death receptors [45]. However, FLIP, like Bax, also binds to Ku70 in an acetylation dependent manner. FLIP binds to the Ku80-binding domain of Ku70. When Ku70 is acetylated at the same two lysines (K539 and K542) that regulate Bax binding, will also dissociate FLIP, triggering FLIP

poly-ubiquitination and degradation. This model suggests that Ku70 regulates apoptosis via the intrinsic pathway, through Bax, and the extrinsic pathway, through caspase 8. Whether FLIP-Ku70 complex that can bind activated Bax is unknown.

Ku80 is a DNA binding partner of Ku70 in the nucleus. Our results also show that Ku80 binds to Ku70 in the cytosol forming complex (figs. 3.2 and 3.4). This complex formation is not affected by HDACI treatment (fig. 3.4). However, unlike cytosolic Ku70, Ku80 does not bind Bax (fig. 3.3). These results are also consistent with the report by Sawada et al showing that Ku80 does not bind Bax, and that Ku70 does not bind Bax and Ku80 simultaneously [1]. Whether the Ku70-Ku80 complex can regulate Ku70-Bax binding remains to be determined.

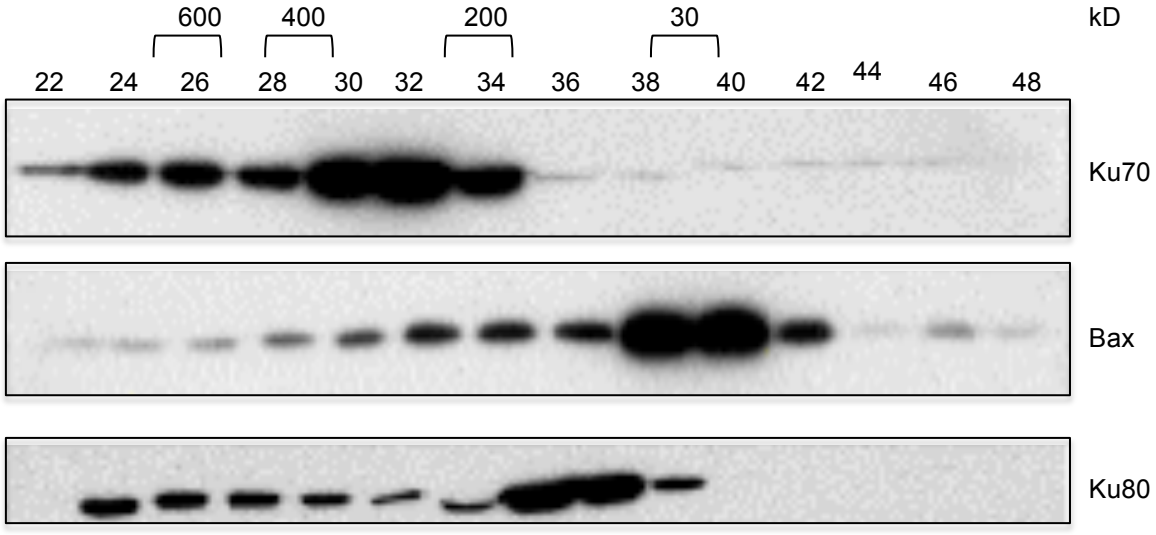

Figure 3.1: Majority of cytosolic Ku70 is found in high molecular weight complexes. Cytosolic SH-SY5Y cell extracts were analyzed by Superdex 200 HR 10/30 column gel filtration chromatography. 20 μl of half a milli-liter fraction was separated by SDS-PAGE and the blot was probed with Ku70, Bax, and Ku80 antibodies.

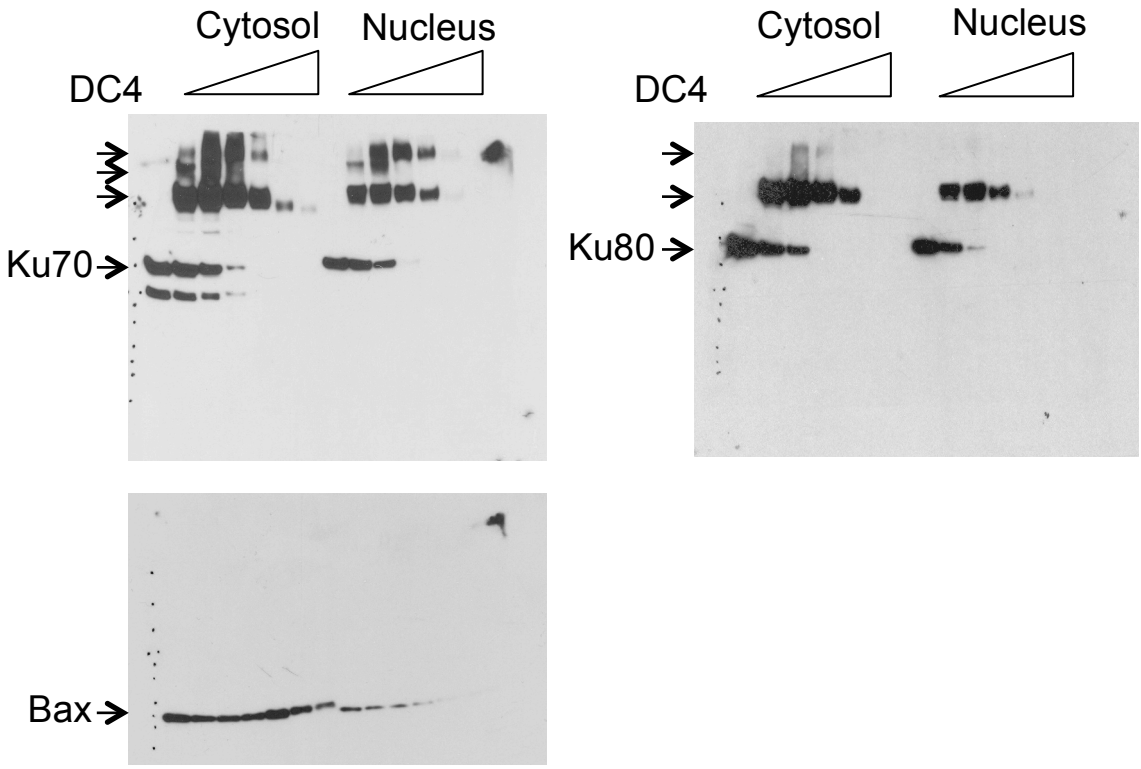

Figure 3.2: Cytosolic Ku70 is found in several high molecular weight complexes in SH-SY5Y cells. Cytosolic extracts were cross-linked using DC4 (0, 0.1, 0.2, 0.4, 0.8, 1.6 mM) as shown. Cross-linked proteins were separated by 10% SDS-PAGE, and the blot was probed with anti-Ku70, anti-Ku80 or anti-Bax antibodies.

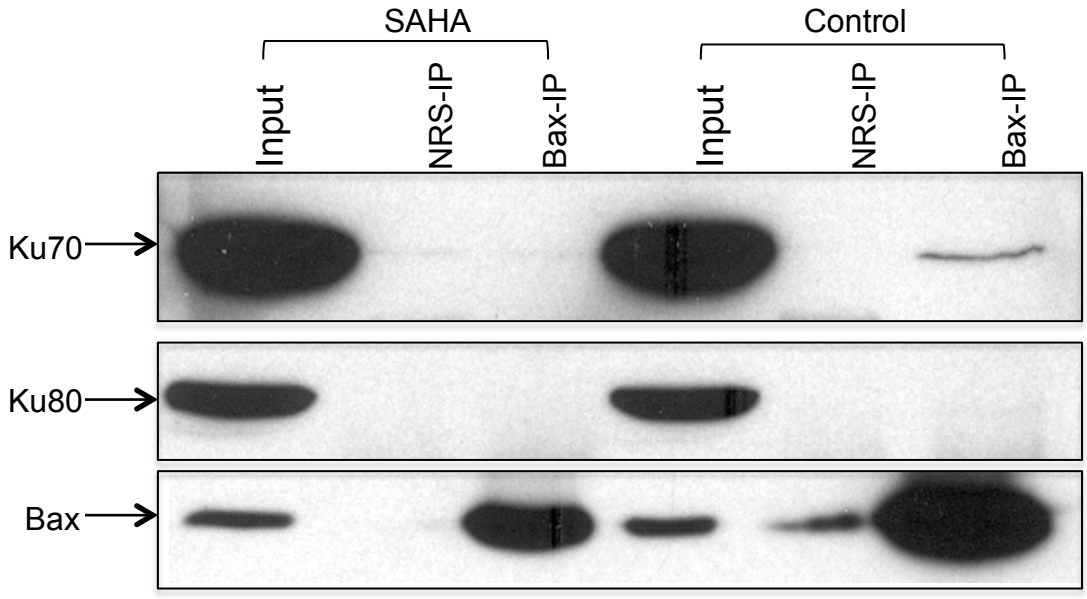

Figure 3.3: Bax binds to Ku70 but not to Ku80. SH-SY5Y cells were treated with SAHA (4 μM) for 48 hours. Control cells received the same volume of DMSO. Cytosolic extracts were immunoprecipitated using an anti-Bax antibody. Normal rabbit serum (NRS) was used as a negative control. Immunoprecipitated complexes were separated by SDS-PAGE and blot was probed with anti-Ku70, anti-Ku80 or anti-Bax antibodies.

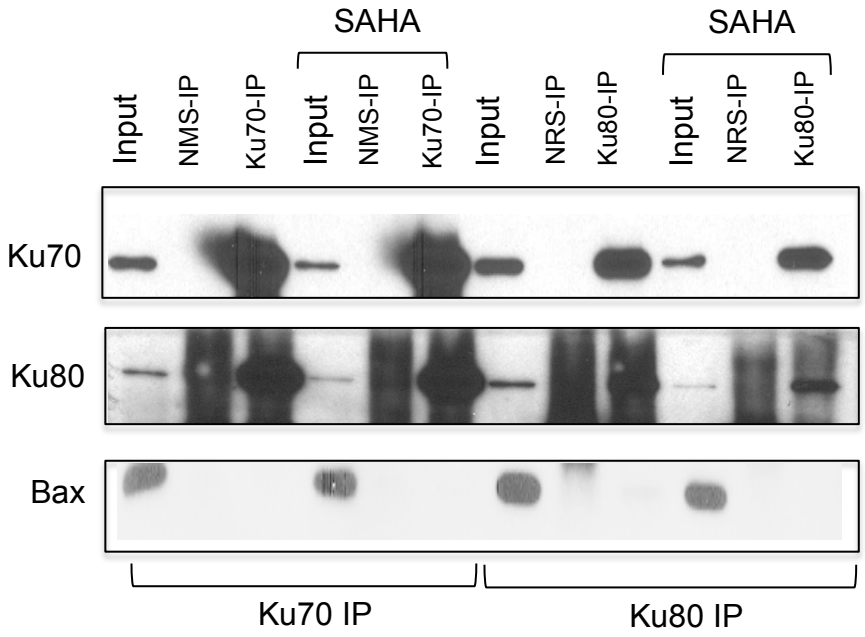

Figure. 3.4: HDAC inhibitor treatment did not alter the binding between cytosolic Ku70 and cytosolic Ku80. SH-SY5Y cells were treated with SAHA (4 μM) for 48 hours. Control cells received the same volume of DMSO. Cytosolic extracts were immunoprecipitated using an anti-Ku70 or anti-Ku80 antibody. Normal mouse serum (NMS) or normal rabbit serum (NRS) was used as negative control, respectively. Immunoprecipitated complexes were separated by SDS-PAGE and blot was probed with anti-Ku70, anti-Ku80, or anti-Bax antibodies.

Chapter 4

Neuronal type Neuroblastoma cells are sensitive to Ku70-depletion induced cell death

4.1 Introduction:

In chapter 3, we have described a model in which, at least in N-type NB cells, Ku70 plays a survival role in regulating Bax's pro-apoptotic activity. We have shown that depletion of Ku70 in SH-SY5Y cells results in Bax-dependent cell death [85]. Studies have demonstrated that, in cells such as HeLa and HEK-293, depleting Ku70 using Ku70 specific siRNA did not induce cell death. However, consistent with our results, Longley et. al. in 2012 have demonstrated that knocking down Ku70 in colorectal cancer cell line (HCT116) induces the cells to undergo apoptosis, and that cells can be rescued by using a pan-caspase inhibitor, z-VAD [45]. These results suggest that there may be at least two cell types in terms of Ku70 regulating Bax activity: one cell type is Ku70-depletion sensitive cells in which Ku70 acts as a survival factor (like SH-SY5Y cells and HCT116 cells); the second cell type is Ku70-depletion insensitive cell type in which Ku70 is not required for survival (like HeLa cells and HEK-293 cells). In this chapter, we investigate the differences in terms of Ku70 regulating Bax activity in the Ku70-depletion sensitive or Ku70-depletion insensitive cell types.

4.2 Results:

Ku70 depletion induces apoptosis specifically in SH-SY5Y cells but not in other cancer cell types

To address whether Ku70 depletion induces cell death, we knocked down Ku70 using Ku70 specific siRNA in several cancer cell lines: N-type NB cells (SH-SY5Y), stromal-type (S-type) NB cells (SHEP-1), Ovarian cancer cells (A2780 and ES-2), and HEK-233T. Ku70 specific siRNA, or scrambled siRNA was transfected in various cell lines. Forty-eight hours after transfection, the cell viability of transfected cells was determined by the trypan blue exclusion assay. Results shown in fig 4.1 indicate that Ku70 depletion reduces cell viability of SH-SY5Y cells but not SHEP-1, ES2, A2780 and HEK-293T cells.

Ku70 depletion reduces cell viability in N-type NB cells.

To determine whether Ku70-depletion induced reduction of cell viability is specific to SH-SY5Y cells, we determined cell viability in three other N-type NB cells (IMR32, KCN-69n, and GOTO) after Ku70 depletion using Ku70 specific siRNA. We used SH-SY5Y as a positive control and SHEP-1 and LA1-5s, two S-type NB cells, as negative controls. Forty-eight hours after siRNA transfection, cell viability was determined by the trypan blue exclusion assay. Results shown in fig. 4.2 indicate that, like for SH-SY5Y cells, cell viability of three other N-type NB cells was reduced by Ku70 specific siRNA transfection while the two S-type NB cells were not affected. These results suggest that the Ku70 requirement for survival is not exclusive to SH-SY5Y cells and maybe a general feature of N-type NB cells.

Ku70, Ku80, and Bax levels are similar in all cell types

Next, we explored the differences between the Ku70-depletion sensitive cells and Ku70 depletion insensitive cells. Here we tested a hypothesis in which Bax requirement for cell death may be different in these Ku70-depletion insensitive cells. One possibility is that Bax may be absent in these cells so Bax is no longer a cell death factor. Thus Ku70 depletion may not have any effect on cell survival. We determined the protein expression of Ku70, Ku80, and Bax in the cytosolic extracts and in the whole cell extracts in SH-SY5Y, SHEP-1, ES2, A2780, and HEK-293T cells. We separated these extracts by SDS-PAGE, and was probed the blot with Ku70, Ku80, or Bax antibodies. β-Tubulin was used as loading control. Fig. 4.3 shows that, in whole cell extracts, the level of Ku70, Ku80, or Bax in all cell lines is similar, except in ES2 in which the Bax level is lower. In the cytosolic extracts, the β-tubulin loading control was uneven; it was higher in SH-SY5Y and SHEP-1 cells, and to some extent also in HEK-293T cells, compared to that of ES2 and A2780 cells. However, the level of Bax followed the same pattern as that of the β-tubulin loading control, being higher in SH-SY5Y, SHEP-1, and HEK-293T cells, but was low in other cell types. The level of cytosolic Ku70 and Ku80 was similar despite the variations in β-tubulin loading control. While we did not conduct a densitometry analyses of these bands, we felt that we can conclude from these results that there are no big variations of the level of Bax and Ku70 in the cytosolic extracts of SH-SY5Y, SHEP-1, and HEK-293T cells, in which their β-tubulin loading control is very similar. These results suggest that the differences between the sensitivity to Ku70 depletion are not due to the differences in the Bax or Ku70 level.

Differences in response to SAHA treatment in different cell types

We have shown previously that SH-SY5Y cells are sensitive to HDAC inhibitor treatments. Using SAHA, TSA (both class I and II HDAC inhibitors) or tubacin (a HDAC6

specific inhibitor), we have shown that these HDAC inhibitors induce Ku70 acetylation and results in Bax dissociation leading to apoptotic Bax-dependent cell death. Here, we explored the differences between Ku70-depletion sensitive and Ku70-depletion insensitive cells in terms of their response to HDAC inhibitor treatment. Cells were treated for 48 hours with SAHA (0, 0.5, 1, 2, and 4 μM) and cell viability was determined by the MTT assay. Results shown in fig. 4.4 shows that the cell viability of SH-SY5Y cell was reduced to 20% while the cell viability of other Ku70-depletion insensitive cells were only reduced to 50% for HEK-293T and A2780, and to 80% (20% reduction) for SHEP-1 cells. Thus, there are clear differences in terms of cell viability response to HDAC inhibitor treatment between Ku70-depletion sensitive and Ku70-depletion insensitive cell types.

Ku70 depletion sensitive and insensitive cells have similar cytosolic Ku70 complex patterns under basal conditions

Using gel filtration chromatography and cross-linking studies, we have shown that in Ku70-depletion sensitive cells, SH-SY5Y cells, Ku70 forms several high molecular weight complexes, and that only a small fraction of Ku70 and Bax bind to each other (fig. 3.1). Similar to SH-SY5Y cells, here we show Ku70-depletion insensitive cells have similar patterns of protein elution in gel filtration chromatography (fig. 4.5, A2870 and HEK293T) and in cross-linking studies (fig 4.6, SHEP-1, ES2, and HEK-293T). These results suggest that there is no difference in Ku70 complex formation amount in these cells.

HDAC inhibitor induces cytosolic Ku70 acetylation but not Ku70-Bax dissociation in Ku70-depletion insensitive cells

We have previously shown that in Ku70-depletion sensitive cells (such as SH-SY5Y cells), HDAC inhibitor treatment induces Ku70 acetylation, Bax dissociation from Ku70, and apoptotic cell death [113]. In fig. 4.4, we have shown that the Ku70-depletion insensitive cells had a partial response to HDAC inhibitor treatment. Thus we next explored whether the Ku70-Bax complex in the Ku70-depletion insensitive cells has similar response to HDAC inhibitor treatment. We first tested whether Ku70 is acetylated in response to HDAC inhibitor in Ku70-depletion insensitive cells. We treated the cells with SAHA (4 μM) for 48 hours. Cytosolic extracts were immunoprecipitated using an anti-acetyl-lysine antibody (Ac-K-103). The immunoprecipitates were separated by SDS-PAGE and the blot was probed with an anti-Ku70 antibody. Results shown in fig. 4.7 indicate that in both, Ku70-depletion sensitive (SH-SY5Y) and Ku70-depletion insensitive cells (SHEP-1, ES2, A2780, and HEK-293T), the cytosolic Ku70 is acetylated following SAHA treatment.

We have shown previously that in SH-SY5Y cells, Ku70 acetylation, either by inhibiting HDAC6 or by depleting HDAC6, resulted in Bax dissociation causing apoptotic cell death [94]. Here, we asked whether Bax is dissociated from Ku70 following HDAC inhibitor treatment in Ku70-depletion insensitive cells. In ES-2 and HEK-293T cells, 48 hours following TSA (10 μM) treatment for ES2 cells or SAHA (4 μM) treatment for HEK-293T cells, we immunoprecipitated Bax using a Bax specific antibody. Normal rabbit serum (NRS) was used as negative control. The immunoprecipitates were separated by SDS-PAGE, and the blot was probed with anti-Bax antibodies or anti-Ku70 antibodies. Results shown in fig. 4.8 show that the Bax antibody immunoprecipitated Ku70 in the cytosolic extracts of both cell types treated with or without

HDAC inhibitor. These results suggest that, unlike those in SH-SY5Y cells, Bax does not dissociate from Ku70 even though Ku70 is acetylated following HDAC inhibitor treatment.

Bax is not activated following HDAC inhibitor treatment in Ku70-depletion insensitive cells

While results shown in fig. 4.7 demonstrated that following HDAC inhibitor treatment Ku70 was acetylated, and that results shown in fig. 4.8 show that Bax did not dissociate from Ku70 in the Ku70-depletion insensitive cells, it is not clear whether Bax was activated while it still bound to Ku70. To address this question, we have used two approaches.

First, we directly asked whether Bax was activated following HDAC inhibitor treatment in Ku70-depletion sensitive cells (SH-SY5Y) and Ku70-depletion insensitive cells (HEK-293T). We used an anti-Bax antibody (6A7) in an immunoprecipitation experiment. This antibody binds to the N-terminal of Bax when Bax is activated [38]. Using this method, we have demonstrated that in control untreated cells, Bax activation is very low in both SH-SY5Y cells and in HEK-293T cells (fig. 4.9). However, 24 hours following SAHA (4 μM) treatment, there was a significant increase in Bax activation in SH-SY5Y cells (increased in 6A7 antibody precipitation). In contrast, there was no increase in 6A7 antibody precipitation in HEK-293T cells. These results suggest that, in Ku70-depletion insensitive cells, HDAC inhibitor treatment did not induce Bax activation.

The second approach we used was to determine the cleavage of pro-caspase 3, a downstream target of Bax activation. We used an anti-caspase 3 antibody that recognizes both pro-caspase 3 and cleaved caspase 3. Both SH-SY5Y cells and HEK-293T were treated with SAHA (4 μM) for 24 hours, equal amount of cytosolic extracts from treated or untreated cells

were separated by SDS-PAGE, and the blot was probed with the anti-caspase 3 antibody. β-tubulin was used as loading control. Results shown in fig. 4.10 demonstrated that while in untreated cytosolic extracts, there was a basal cleavage of pro-caspase 3 in both cell types, there were no significant differences compared to the untreated and treated extracts in HEK-293T, In contrast, in SH-SY5Y cells, pro-caspase 3 level was markedly reduced in SAHA treated cytosolic extracts. These results suggests that, as predicted, HDAC inhibitor treatment of SH-SY5Y cells activated Bax, resulted in Bax translocation to the mitochondria, leading to the activation of caspase 3 (cleavage of pro-caspase 3). However, in HEK-293T cells, HDAC inhibitor treatment did not activate Bax; thus Bax did not translocate into the mitochondria, and did not activate pro-caspase 3.

4.3 Discussion

The focus of this chapter was to address a fundamental question in terms of Ku70-Bax complex regulation: Do all cells behave the same way in regulating Ku70-Bax binding? In chapter 3, using N-type NB cells, SH-SY5Y, we have proposed a model in which Ku70 is acting as a survival factor in protecting the sporadic activation of Bax. We have also provided evidence showing that there may be another level of regulation in which factors affecting Ku70 dissociation from other factors may also be important for the survival of the cells. In this chapter, we have provided evidence showing that Ku70 does not serve as a survival factor in all cell types tested. When Ku70 is acting as a survival factor, such as in SH-SY5Y cells, depletion of Ku70 results in cell death. However, when Ku70 is not acting as a survival factor such as in SHEP-1, A2780, ES2, HEK293T cells, Ku70 depletion does not result in cell death.

Previous results have indicated that Ku70 is a desensitizer for Bax-activated cell death [105, 115]. Increasing level of Ku70 reduces the effect of cell death inducing agent, such staurosporine [1], which activates Bax. Thus, cytosolic Ku70 may have at least two different functions in different cells. One is that Ku70 is acting as a survival factor. Without it, the cell will die, as we see in SH-SY5Y cells. Another function of Ku70 may be to block Bax when Bax is activated by other means. However, in these cells, Ku70 does not act as a survival factor because depleting Ku70 did not induce cell death. There must be an additional mechanism that suppresses Bax-induced cell death at basal level.

To distinguish the Ku70-depletion sensitive from the Ku70-depletion insensitive cell types, as our results suggest, is to see whether Ku70 is associated with Bax when Ku70 is acetylated. One established model is that Ku70-Bax binding is regulated by the ability of Ku70 to be acetylated. Ku70 acetylation is regulated by CBP and HDAC6. Therefore, this model

suggests that in order for Ku70 to be a survival factor binding to Bax and inhibiting Bax activation, Ku70 needs to be actively deacetylated.

Previously in our lab, using SH-SY5Y cells, we have provided evidence to support this hypothesis [102, 113]. However, in Ku70-depletion insensitive cells, using HDAC inhibitor SAHA, we have shown that even though cytosolic Ku70 is acetylated (fig. 4.7), Ku70 remains bound to Bax (fig. 4.8). These results suggest that in these Ku70-depletion insensitive cells, Ku70 acetylation may not be the sole factor that regulates Bax binding; there are some other factors controlling Bax binding to Ku70.

However, one may argue that our experimental procedures have over-estimated the level of Ku70 acetylation. It is because we immunoprecipitated cytosolic extracts using an anti-acetyl-lysine antibody, and the presence of Ku70 in these extracts were verified using immunobloting using an anti-Ku70 antibody. It is possible that the presence of Ku70 in the cytosolic extracts was the result of Ku70 association with other acetylated factors that were immunoprecipitated by the anti-acetyl-lysine antibody. This is a distinct possibility. We have attempted to immunoprecipitate Ku70 from cytosolic extracts using Ku70 specific antibody, and then determined Ku70 acetylation in immunobloting using an anti-acetyl-lysine antibody. The results of these experiments were variable and the signal was very weak. One possibility is that the total level of acetylated Ku70 is very low. It may be that only Ku70 that binds to Bax gets acetylated. Therefore, the percentage of Ku70 that is acetylated in total immunoprecipitated Ku70 may be lower than or at the limit of the detection of immunobloting.

One means to determine acetylation of endogenous proteins is to label the cells with radio-labeled acetyl-CoA followed by immunoprecipitation using antibodies. However this method requires very high level of radio-labeled acetyl-CoA, and depending on the number of

acetylated lysines present, the signal may be low and it may take a long time (a month or more) to see any signal.

It has been reported that Ku70 is at least acetylated on 8 lysines (K282, K331, K338, K539, K542, K544, K553, and K556) [89, 116]. When two lysines K539 and K542 are acetylated, Bax is dissociated from Ku70. We have attempted to raise antibodies against these two acetylated-lysines. However, we were unsuccessful due to low immunogenic properties of these sites.

Another difference between Ku70-depletion sensitive and Ku70-depletion insensitive cells is their response following HDAC inhibitor treatment. We have shown previously that HDAC inhibitor treatment of SH-SY5Y cells results in Bax dissociation from Ku70. The dissociated Bax enters mitochondria, releasing cytochrome c, triggering cell death. However, the cell viability of the Ku70-depletion insensitive cells following HDAC inhibitor treatment is almost half of the response observed in the Ku70-depletion sensitive cells. Furthermore, HDAC inhibitor treatment of Ku70-depletion insensitive did not follow the classical pathway of apoptosis: Bax did not dissociate from Ku70, Bax was not activated (fig. 4.9), and the pro-caspase 3 was not cleaved (fig. 4.10). These results suggest that HDAC inhibitor treatment of Ku70-depletion insensitive cells may trigger a different cell death pathway causing cell death.

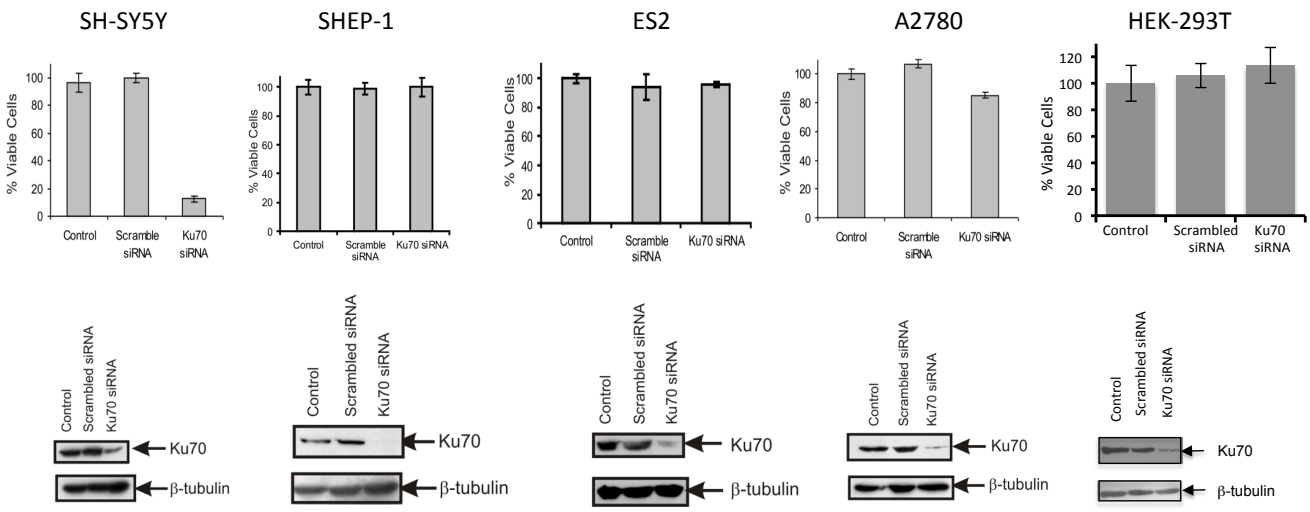

Figure 4.1: Ku70 depletion reduces cell viability in SH-SY5Y cells but not in SHEP-1, ES2, A2780, and HEK-293T cells. Cells received two sequential transfections of Ku70 specific siRNA. Scrambled siRNA was used as negative control. Two days after last transfection, cell viability was determined by trypan blue exclusion assay. The degree of Ku70 depletion was determined by SDS-PAGE using anti-Ku70 antibody. β-tubulin was used as loading control.

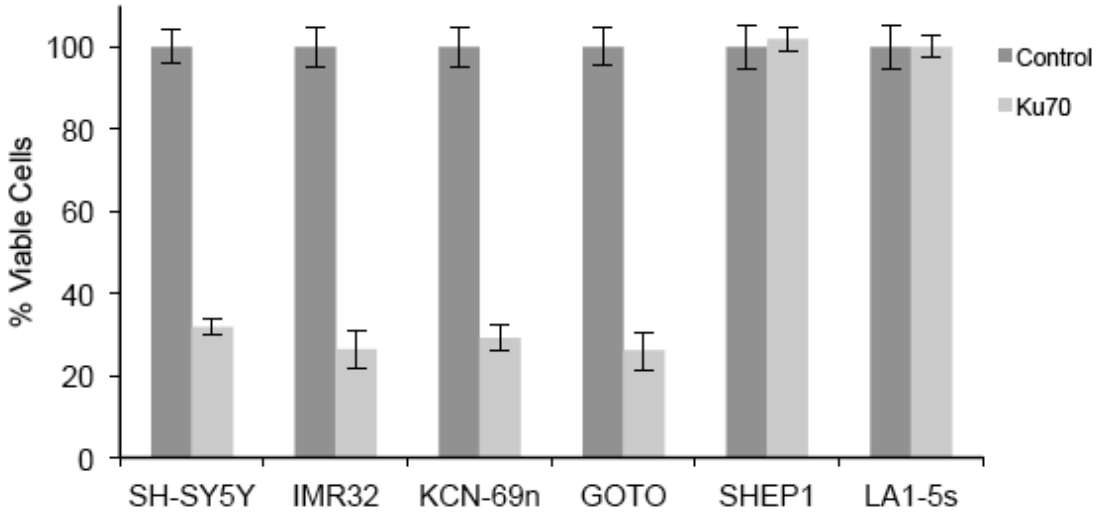

Figure 4.2: Ku70 depletion reduces cell viability of neuronal neuroblastoma cells. Cells received two sequential transfections of Ku70 specific siRNA. Scrambled siRNA was used as negative control. Two days after last transfection, cell viability was determined by trypan blue exclusion assay.

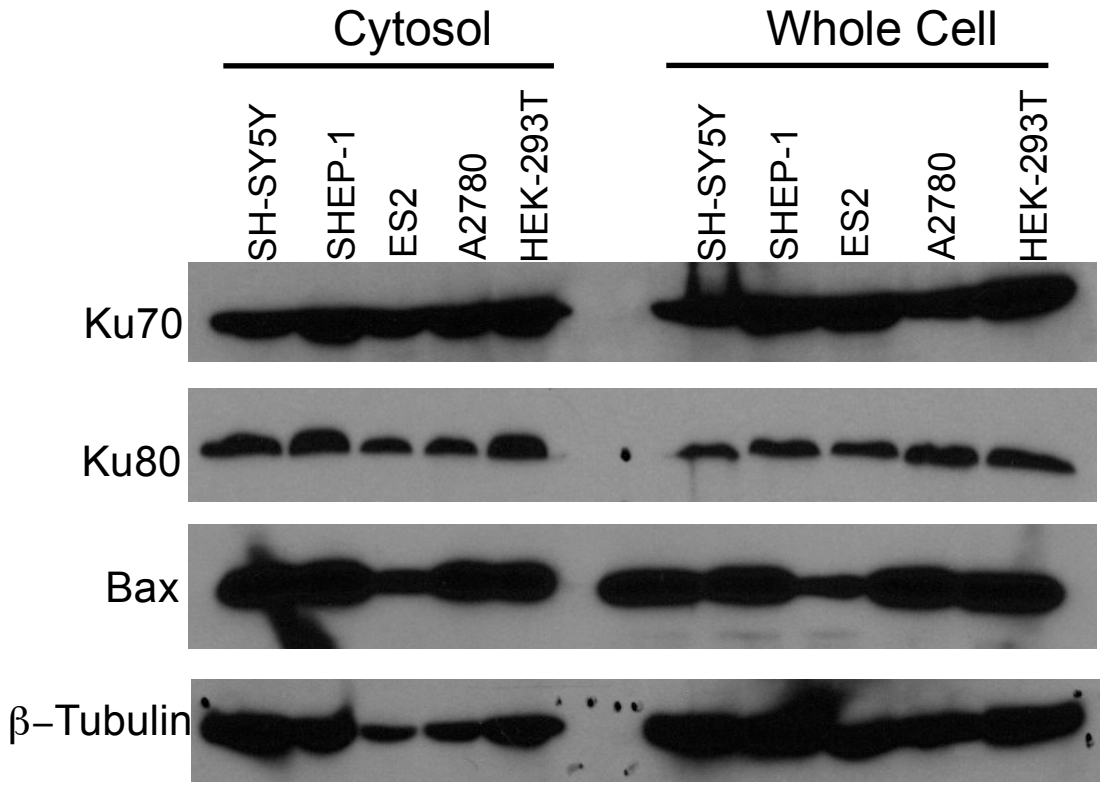

Figure 4.3: Ku70-depletion sensitive or insensitive cells have similar level of Ku70, Ku80 or Bax. Cytosolic extracts or whole cell extracts were separated by SDS-PAGE and the blot was probed with anti-Ku70, anti-Ku80, or anti-Bax antibodies. β-tubulin was used as loading control.

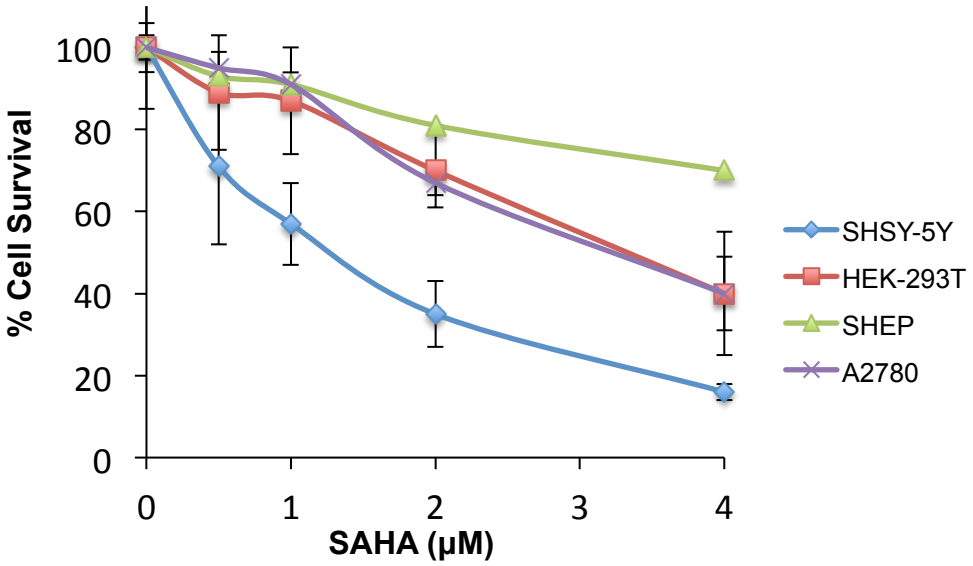

Figure 4.4: HDAC inhibitor treatment is less effective in reducing cell viability in Ku70-depletion insensitive cells compared to Ku70-depletion sensitive cells. Cells were plated into 96 well plates. One day later, they were treated with various concentrations of SAHA as shown. There were at least three wells per concentration. 48 hours after SAHA treatment, cell viability was determined by MTT. The results of the MTT assay were expressed as percent of DMSO only treated control in MEAN±SD.

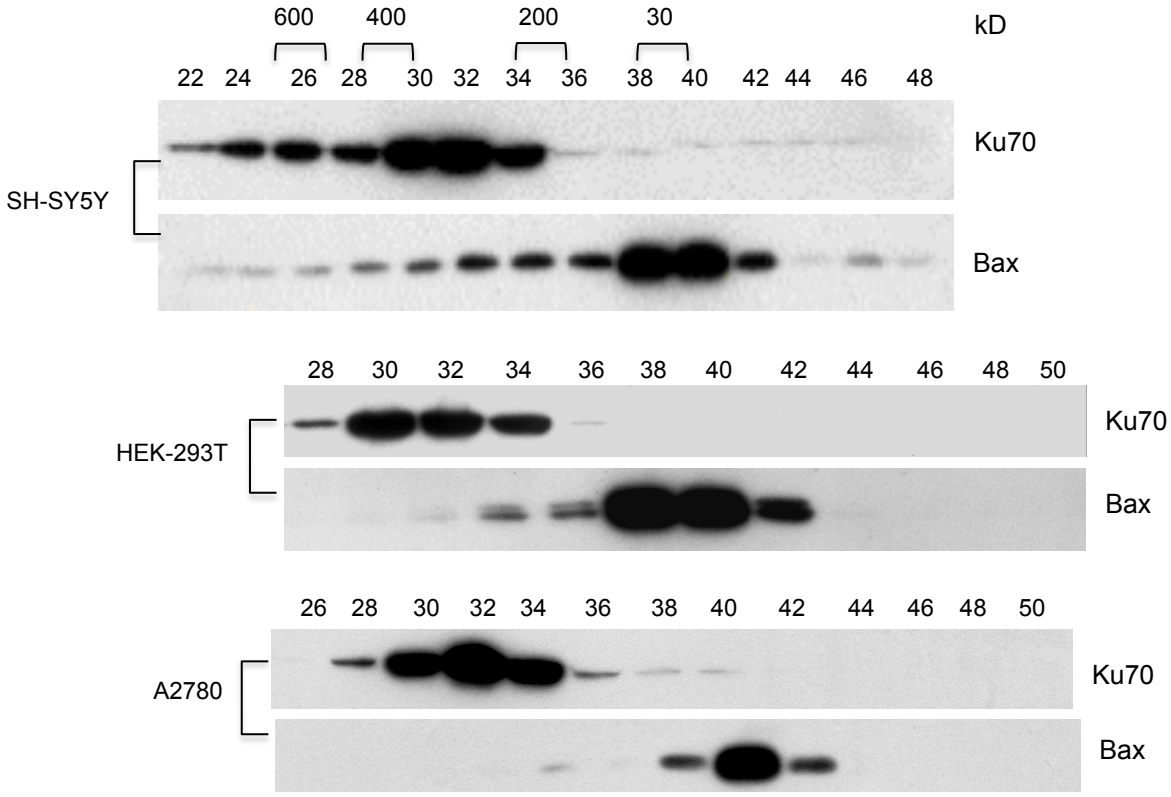

Figure 4.5: Ku70 and Bax have similar elution pattern in gel filtration chromatography in SH-SY5Y, HEK-293T, or A2780 cells. Cytosolic SH-SY5Y, HEK-293T, or A2780 cell extracts were analyzed by Superdex 200 HR 10/30 gel filtration chromatography. 20 µl of half a milliliter fraction was separated by SDS-PAGE and the blot was probed with anti-Ku70, anti-Bax, or anti-Ku80 antibodies.

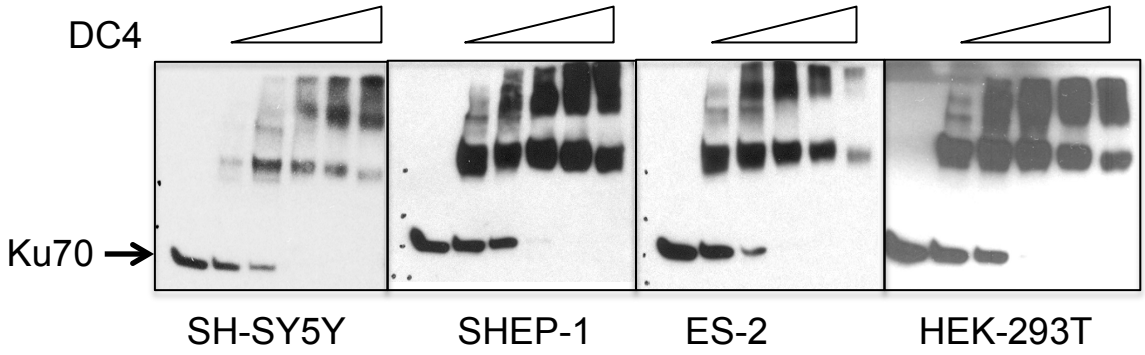

Figure 4.6: SH-SY5Y, SHEP-1, ES2, and HEK-293T cells have similar Ku70 complex pattern in cross-linking studies. Cytosolic extracts were cross-linked using various DC4 concentrations (0, 0.1, 0.2, 0.4, 0.8, 1.6 mM) as shown. Cross-linked proteins were separated by 10% SDS-PAGE, and the blot was probed with anti-Ku70 antibodies.

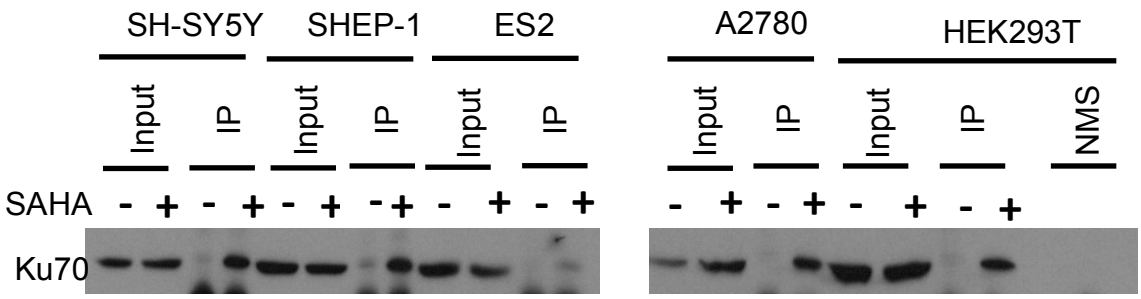

Figure 4.7: Cytosolic Ku70 is acetylated by HDAC inhibitor treatment in cells. Cells were treated with SAHA (4 μM) for 48 hours. Control cells received the same volume of DMSO. Cytosolic extracts were immunoprecipitated using an anti-acetyl-lysine antibody. (Ac-K-103). Normal mouse serum (NMS) using HEK-293T cytosolic extracts was used as negative control. Immunoprecipitated complex was separated by SDS-PAGE and the blot was probed with anti-Ku70 antibodies.

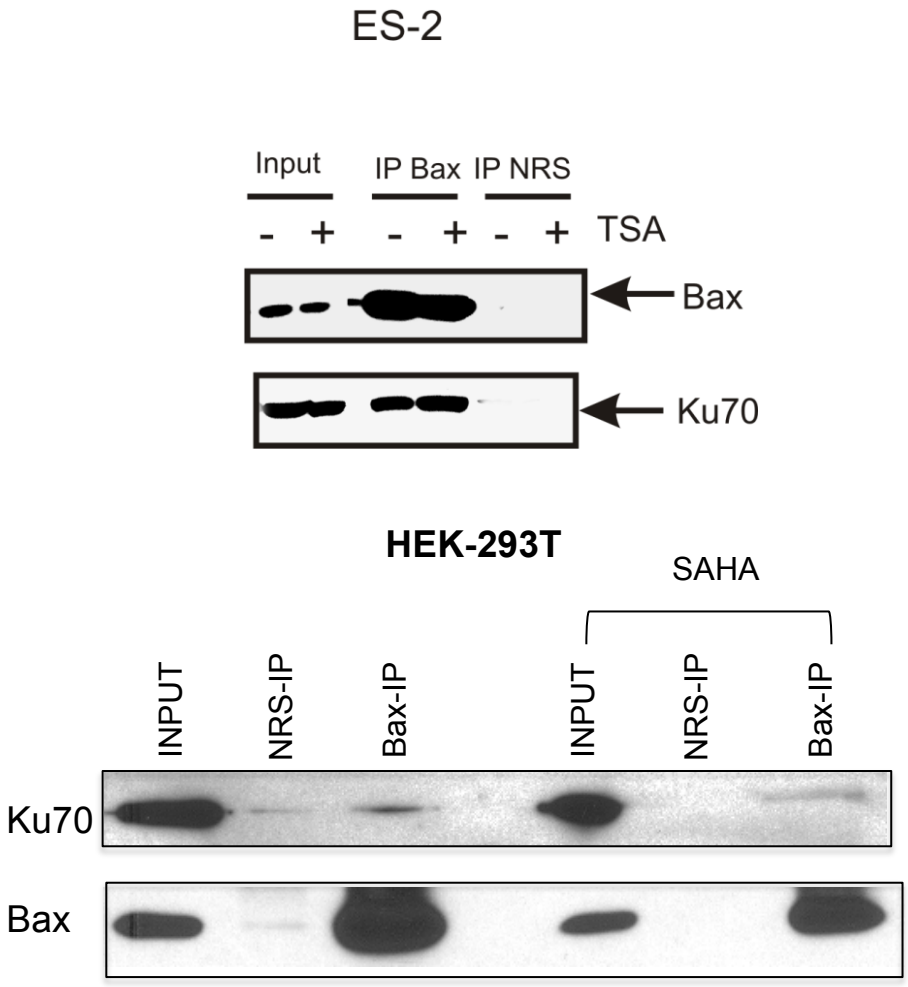

Figure 4.8: Bax is not dissociated from Ku70 following HDAC inhibitor treatment in ES2 and HEK-293T cells. ES2 cells were treated with TSA (10 μM) for 48 hours. HEK-293T cells were treated with SAHA (4 μM) for 48 hours. Cytosolic extracts were immunoprecipitated using anti-Bax antibodies. Normal rabbit serum (NRS) was used as control. Immunocomplexes were separated by SDS-PAGE and the blot was probed with anti-Bax or anti-Ku70 antibodies.

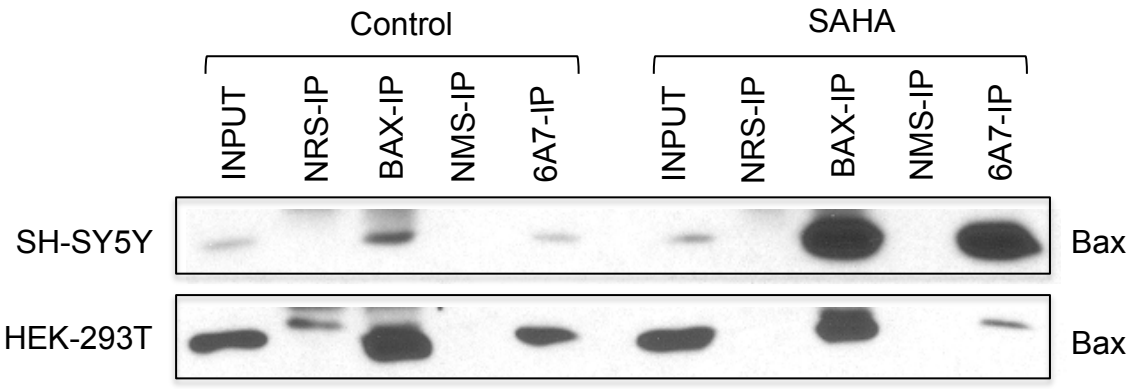

Figure 4.9: Bax is activated following HDAC inhibitor treatment in SH-SY5Y cells but not in HEK-293T cells. SH-SY5Y or HEK-293T cells were treated with SAHA (4 μM) for 24 hours. Control cells received only DMSO. Cytosolic extracts were immunoprecipitated using an anti-Bax antibody or an anti-activated Bax antibody (6A7). Normal rabbit serum (NRS) or normal mouse serum (NMS) was used as control. Immunoprecipitated complex was separated by SDS-PAGE and the blot was probed with anti-Bax antibodies.

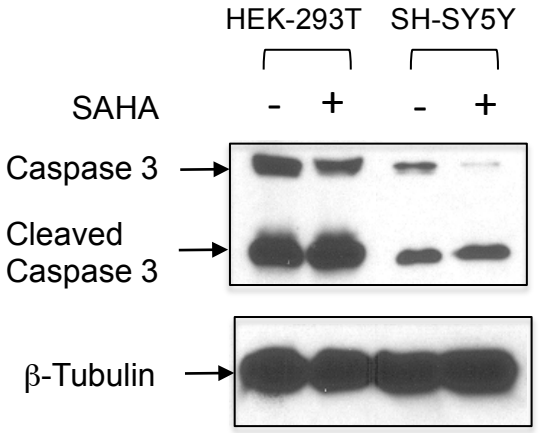

Figure 4.10: Caspase 3 is activated following HDAC inhibitor treatment in SH-SY5Y cells but not in HEK-293T cells. SH-SY5Y or HEK-293T cells were treated with SAHA (4 μM) for 24 hours. Cytosolic extracts were analyzed by SDS-PAGE, and the blot was probed with anti-caspase 3 antibodies. β-tubulin was used as loading control.

Chapter 5

Conclusion and Future Directions

In this thesis project, I have investigated two fundamental questions of how Ku70 regulates Bax's activities in cells. The first question was whether all Bax bound to Ku70; the second question was whether the Ku70 acting as a survival factor model was applicable to other cell types.

We have addressed the first question in Chapter 3. We have provided evidence showing that only a small fraction of Bax and a small fraction of Ku70 bind to each other. The majority of Bax is monomeric. This raises an important question: what makes the small amount of Bax bind to Ku70? What is the difference between Ku70-bound Bax compared to the monomeric Bax? Our hypothesis is that Ku70 acts as a survival factor. At the basal level, Ku70 binds to aberrantly activated small amount of Bax, rendering Bax to be inactive and blocking Bax's cell death function. This model suggests that Bax needs to be activated when it binds to Ku70. As discussed above in the Chapter 1 that the N-terminal of Bax is exposed when Bax is activated. Is it possible that the N-terminally exposed, activated Bax binds to Ku70? Studies have shown that Bax lacking 1-54 amino acids does not bind to Ku70 [105]. This suggests that Bax's Ku70 binding domain may be between 1-53 amino acids. However, it is possible that lacking 1-54 amino acids in Bax may result in a conformational change of Bax molecule such that Bax can no longer bind Ku70. There is no in vitro binding data to verify where the Ku70-binding domain of Bax is.

Another possibility of how Bax can bind to Ku70 is that the Ku70-bound Bax may be post-translationally modified. Bax is known to be phosphorylated and ubiquitinated. However, there is no evidence that these modifications affect its binding to Ku70. To answer the question on what makes Bax binding to Ku70, one may need to use an unbiased approach to analyze Ku70-bound Bax using mass spectrometry. Sequential immunoprecipitations will be used to pull down Ku70-bound Bax. The first immunoprecipitation would use anti-Ku70 antibodies to precipitate Bax-bound Ku70, and the second would use anti-Bax antibodies to precipitate Bax from the Bax-Ku70 complex. The precipitated Bax will be analyzed by mass spectrometry to determine post-translational modifications of Bax. Targeting Bax modifications that affect it's binding to Ku70 may be a new therapeutic approach in inducing cell death.

In chapter 3, we have also provided evidence showing that the non Bax-bound Ku70 forms several high molecular weight complexes in the cytosol. Most importantly, there is no monomeric cytosolic Ku70. The Ku70 acting as a survival factor model predicts that because there is no free Ku70 that can bind to more activated Bax. Ku70 needs to be from somewhere. Ku70 that is capable to bind Bax is either released from Ku70-containing complexes, or alternately, Ku70 that is in complex with other factors may still be able to bind to activated Bax. Thus there is another level of regulation of the availability of Ku70 to bind Bax. To understand what regulates Ku70 binding to other factors, we need to identify factors that bind to cytosolic Ku70. As discussed in the Introduction chapter, several studies have demonstrated that Ku70 binds to other factors in the cytosol. We have also shown that Ku70 binds Ku80, its binding partner in the nucleus. However, how the binding of Ku70 to these factors affects Ku70-Bax binding remains unclear. It will be interesting to see whether the known Ku70-associated proteins like FLIP or caveolin-1 form complex with Ku70 in cross-linked studies. We have also proposed to use Ku70 immunoprecipitation followed by mass spectrometry to identify additional

cytosolic Ku70 binding proteins. This will be an unbiased approach identifying factors in Ku70-depletion sensitive and Ku70-depletion insensitive cells.

In our Ku70 acting as a survival factor model we have proposed that, at the basal level, cytosolic Ku70 acts as a survival factor suppressing Bax-mediated cell death. However, in contrast, in the nucleus, at the basal level, Ku70 does not bind DNA. Following DNA damages, like following irradiation, Ku70 in the cytosol as well as in the nucleus is acetylated [88]. At this time, in the nucleus, Ku70 dimerizes with Ku80 and binds to DSB-DNA to start the NHEJ repair process. Thus, nuclear Ku70 is acting as a pro-survival factor to repair DNA damage, protecting the cells from dying. However, simultaneously, in the cytosol, Ku70, upon acetylation, dissociates from Bax, allowing Bax to translocate to mitochondria triggering cell death, at least in Ku70-depletion sensitive cells. It is a pro-apoptotic process, opposing what Ku70 is trying to achieve in the nucleus. How can it be that one protein has two functions opposing each other at the same time? Do cytosolic Ku70 communicate with nuclear Ku70? Do cytosolic Ku70 and nuclear Ku70 regulate each other? If they do, what is the mechanism? We have previously shown that following irradiation in SH-SY5Y cells, the level of cytosolic Ku70 reduces while nuclear Ku70 level increases. This may mean that following irradiation, Ku70 is translocating from the cytosol to the nucleus. It is also possible that cytosolic Ku70 following irradiation is being degraded, and the synthesis of nuclear Ku70 is increased to meet the demand of DNA repair to protect the cells from dying. Currently, to what extent that cytosolic Ku70 affects the DNA-repair function of Ku70 in the nucleus is not known. Is it possible that Ku70 translocation following radiation depends on the degree of DNA damage caused by radiation, such that larger DNA damage will allow cytosolic, acetylated Ku70 to translocate into the nucleus, reducing the DNA-repair activity and allowing the cell to die? If this model is correct, how is it regulated? Currently, answers to these questions remain unknown.

The second question that I have addressed in this project was whether the Ku70 acting as a survival factor model was applicable to other cell types. We have addressed this question in Chapter 4. We have provided evidence showing that there is at least two types of Ku70 responding cells: one is Ku70-depletion sensitive cells, which require Ku70 for survival, and one is Ku70-depletion insensitive cells, which do not require Ku70 for survival. While we have shown that Bax binding to Ku70 is regulated by the acetylation status of Ku70 in the Ku70-depletion sensitive cells, in the Ku70-depletion insensitive cells, acetylation of Ku70 does not dissociate Bax from Ku70, suggesting that there is another factor (or factors) that regulates Ku70-Bax binding. The requirement of Ku70 acetylation in regulation of Ku70-Bax binding in the Ku70-depletion insensitive cells is currently unknown. To answer this question, it may require sequential immunoprecipitations using Bax antibodies following by Ku70 antibodies (or vice versa) to pull down complexes that contain both Bax and Ku70 in the Ku70-depletion insensitive cells. The immunocomplex can be analyzed by mass spectrometry to identify unknown factors.

An important question raised by our results shown in chapter 4 is what are the markers that can distinguish between the Ku70 depletion sensitive cells and the Ku70-depletion insensitive cells? Is it neuronal cells (N-type cells), which are Ku70-depletion sensitive versus non-neuronal cell types, which are Ku70-depletion insensitive? To our knowledge, there is no neuronal cell specific death pathway. Some studies have shown that young neurons are sensitive to cell death signal compared to more mature neurons [117-119]. In some studies, N-type NB cells are considered to represent immature neuronal cell type [120]. Thus, they may be more sensitive to cell death signals. However, Longley et. al. in 2012 have demonstrated that knocking down Ku70 in a colorectal cancer cell line (HCT116) also induces cell death [45], suggesting that not only neuronal cells are sensitive to cell death in response to Ku70 depletion. We have also attempted to answer this question by studying two neuronal cell types, PC-12 and neuro-2A cells (a gift from Dr. Michael D. Uhler). PC-12 is a rat pheochromocytoma cells, and

Neuro-2A is a mouse neuroblastoma cell line. We first determined the Ku70 level in these cells. However, the reason is still unknown to us, we were not be able to measure Ku70 in the extracts of these cells consistently. Even if we could observe Ku70 on a western blot, the signal was very faint. But, we could measure other proteins, like tubulin on the same blot. We also checked whether the Ku70 antibodies that we used were specific for rat and mouse Ku70. We purchased a new Ku70 antibody for this purpose. However, we still failed to detect Ku70 in these cells. It would be interesting to investigate whether different extraction procedures would produce more reliable detection of Ku70 in these cells.

It is important to identify markers to determine the sensitivity to Ku70 depletion in cells. One can imagine that if we can identify cancer cells that are Ku70-depletion sensitive, we may be able to target the Ku70-Bax complex as a therapeutic end point. Potential therapeutics will be developed to activate Bax causing cell death in these cells by separating Bax from Ku70. Studies have shown that a 5-residue peptide (VPMLK), corresponding to the Ku70 sequence between 578-582, competes with Ku70 to bind Bax blocking Bax-mediated cell death [105]. This reagent has been shown to block Bax's cell death activities [105]. However, there is no equivalent reagent developed to bind and compete with Bax for Ku70 binding resulting in free Bax and causing cell death. This potential reagent may be important in the treatment of cancer cells that are Ku70-depletion sensitive, such as NB cancer and colorectal cancer.

In summary, the results of my thesis re-define the Ku70-regulating of Bax activity model. While we have definitively shown that only a small amount of Bax binds to a small amount of Ku70 in cells, how Ku70 regulation of Bax activity depends on the response of the individual cell type to Ku70-depletion. In Ku70-depletion sensitive cells, in which Ku70-depletion triggers cell death, Bax binding to Ku70 is regulated by Ku70 acetylation such that when Ku70 is acetylated, Bax is dissociated from Ku70, triggering cell death. For this model, we have shown that the

availability of Ku70 to bind to increased amount of activated Bax is also a factor that regulates Bax activity in cells.

In the Ku70-depletion insensitive cells, in which Ku70-depletion does not trigger cell death, Bax binding to Ku70 is not regulated by Ku70 acetylation. This finding is in contrast with the current model reported in the literature. We hypothesized that there is another factor (or factors) that regulates Ku70 and Bax binding.

Our results reported in this thesis raise several interesting questions for future experimentations. I have already described these questions in more detail above in this chapter. I will summarize the future directions below:

1. What is the characteristic of the small amount of Bax that binds to Ku70?
2. What are other factors that bind to cytosolic Ku70 and how do these Ku70-containing complexes regulate Ku70-Bax binding?
3. Do cytosolic Ku70 and nuclear Ku70 regulate each other's activity?
4. In Ku70-depletion insensitive cells, what factor or factors regulate the binding between Ku70 and Bax?
5. What are the markers to distinguish between Ku70-depletion sensitive cells and Ku70-depletion insensitive cells?
6. Can we generate a reagent that can compete with Bax for Ku70 binding resulted in freeing Bax to kill cells? This reagent will be useful to trigger cell death in tumor cells in which they are Ku70-depletion sensitive.

Regulating Bax activity is critical in maintaining cell survival. Triggering Bax release from Ku70-Bax complex in either Ku70-depletion sensitive or Ku70-depletion insensitive cells may

serve as a therapeutic endpoint in increasing cell death in these cells. Knowledge gained from answering the proposed questions will be beneficial in this approach.

Appendices

Appendix A: A copy of a peer-reviewed article published in the Molecular Cancer Research in 2013 (Vol. 11 pg.173-181) entitled “CREB-binding protein regulates Ku70 acetylation in response to ionization radiation in neuroblastoma” is included. I was the second name author of the article.

DNA Damage and Repair

Molecular Cancer Research

CREB-Binding Protein Regulates Ku70 Acetylation in Response to Ionization Radiation in Neuroblastoma

Chitra Subramanian[1], Manila Hada[2,3], Anthony W. Opipari Jr[2], Valerie P. Castle[1], and Roland P.S. Kwok[2,3]

Abstract

Ku70 was originally described as an autoantigen, but it also functions as a DNA repair protein in the nucleus and as an antiapoptotic protein by binding to Bax in the cytoplasm, blocking Bax-mediated cell death. In neuroblastoma (NB) cells, Ku70's binding with Bax is regulated by Ku70 acetylation such that increasing Ku70 acetylation results in Bax release, triggering cell death. Although regulating cytoplasmic Ku70 acetylation is important for cell survival, the role of nuclear Ku70 acetylation in DNA repair is unclear. Here, we showed that Ku70 acetylation in the nucleus is regulated by the CREB-binding protein (CBP), and that Ku70 acetylation plays an important role in DNA repair in NB cells. We treated NB cells with ionization radiation and measured DNA repair activity as well as Ku70 acetylation status. Cytoplasmic and nuclear Ku70 were acetylated after ionization radiation in NB cells. Interestingly, cytoplasmic Ku70 was redistributed to the nucleus following irradiation. Depleting CBP in NB cells results in reducing Ku70 acetylation and enhancing DNA repair activity in NB cells, suggesting nuclear Ku70 acetylation may have an inhibitory role in DNA repair. These results provide support for the hypothesis that enhancing Ku70 acetylation, through deacetylase inhibition, may potentiate the effect of ionization radiation in NB cells. *Mol Cancer Res; 11(2); 173–81. ©2012 AACR.*

Introduction

Ku70 was first characterized as an autoantigen and, subsequently, it was also identified to be a nuclear DNA-binding component of the nonhomologous end joining (NHEJ) DNA-repair complex (1). When dimerized with Ku80, Ku70 binds to the broken end of DNA double-strand breaks (DSB; ref. 2). However, someother studies have also shown that Ku70 is also present in the cytoplasm (3). To date, 1 described function of cytoplasmic Ku70 is to bind Bax, an apoptotic protein, thereby blocking Bax-mediated cell death. The binding between Ku70 and Bax is regulated by Ku70 acetylation (4). We have previously shown that inhibiting deacetylase activity in neuroblastoma (NB) cells increases Ku70 acetylation, resulting in Bax release that triggers Bax-dependent cell death (5). Our studies further indicated that cytoplasmic Ku70 plays an important role in NB cell survival as Ku70 knock down or increased Ku70 acetylation by inhibiting HDAC activity induces NB cell death mediated by Bax (6).

In the nucleus, Ku70 (7) when dimerized with Ku80, binds and bridges 2 proximal broken DNA ends and facilitates the repair machinery through a cascade of reactions that involve DNA-dependent protein kinase and DNA ligase IV (8, 9). Ku70 plays a critical role in this DNA repair activity as even partial knock down of Ku70 has been shown to enhance the radiosensitivity of human MCF10A cells (10). Moreover, murine embryonic stem cells deficient in Ku70 are sensitive to radiation and have V(D)J recombination defects and deficiencies in DNA binding (11). In cells with targeted deletion of Ku70, the Ku80 partner is unstable as is the Ku70 partner in Ku80-deficient cells (11, 12). The 2 DNA-binding domains of Ku70 present in the NH_2 and COOH termini are required for high-affinity DNA binding. In addition, the COOH terminal of Ku70 also binds to Bax and prevents apoptotic translocation of Bax to the mitochondria (13). Thus, Ku70 mediates cytoprotective functions through 2 distinct mechanisms: DNA repair in the nucleus and blocking Bax-mediated cell death in the cytoplasm.

Although we and others have shown that acetylation regulates the binding between cytoplasmic Ku70 and Bax (14), the effect of Ku70 acetylation in the nucleus remains unclear. We have previously shown that, in NB cells, acetylation of Ku70 by CBP at lysines 539 and 542 resulted in Bax release from Ku70, followed by Bax translocation to mitochondria (5). Computer docking analysis indicated the presence of multiple lysine residues that form a positively charged lining for interaction with broken DNA ends at the DNA-binding cradle of Ku70 (4, 15). Additional studies carried out in prostate cancer cells using site-directed mutagenesis to replace the lysine residues at K282, K338, K539, or K542 with glutamine showed that, in addition to the above-mentioned lysine residues, namely K539 and K542, 2

Authors' Affiliations: Departments of [1]Pediatrics, [2]Obstetrics and Gynecology, and [3]Biological Chemistry, University of Michigan, Ann Arbor, Michigan

Corresponding Author: Roland P.S. Kwok, University of Michigan, 6428 Medical Science 1, 1500 W. Medical Center Drive, Ann Arbor, MI, 48109. Phone: 734-615-1384; Fax: 734-936-8617; E-mail: rkwok@umich.edu

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other lysine residues, K282 and K338, also take part in binding broken-end DSB DNA (16). The fact that the K539 and K542 acetylation by CBP are responsible for Bax-dependent cell death in NB cells and the same lysine residues are involved in binding to broken-end DSB DNA prompted us to investigate the role of Ku70 acetylation by CBP in response to ionizing radiation-induced DNA damage.

Our results show that ionizing radiation does not affect Ku70 expression in NB cells but ionizing radiation induces Ku70 redistribution from the cytoplasm to the nucleus. When NB cells are subjected to ionizing radiation, the more aggressive neuroblastic (N-type) NB cells undergo cell death while the less aggressive stromal (S-type) NB cells show ionizing radiation resistance. Moreover, the DNA repair activity, as measured by phosphorylated H2AX (γ-H2AX) expression and by the Comet assay, is more efficient in the S-type cells compared with the N-type cells. The possibility that increased acetylation of Ku70 might block Ku70 DNA-binding activity necessary for NHEJ repair, together with previous work showing N-type cells express higher levels of CBP, led us to hypothesize that Ku70 acetylation by CBP may play a critical role in ionizing radiation-induced killing of N-type NB cells. This model is supported by evidence that knocking down CBP in N-type cells led to a reduction of Ku70 acetylation, increased DNA-repair activity, and NB cell survival following ionizing radiation. These results suggest a critical role for CBP in Ku70 acetylation following ionizing radiation-induced DNA damage in NB cells and provide further support for the development of NB therapeutic strategies that target Ku70 acetylation.

Materials and Methods

Cell culture and irradiation

Human NB cell lines SH-SY5Y, IMR32, SH-EP1, and the fibroblast cell line IMR90 were cultured in modified Eagle's medium (MEM) supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin, and the cells were maintained at 37°C in a humidified 5% CO_2 incubator. The cells were irradiated over the clinically relevant dosage ranging from 0 to 20 Gy using Philips 250 orthovoltage unit at the Irradiation Core of the University of Michigan Cancer Center.

Cell viability assay

The viability of the human NB cell lines SH-SY5Y, SH-EP1, IMR32, and the fibroblast cell line IMR 90 was determined 24 and 48 hours after exposure to ionizing radiation by MTT or sulforhodamine assays as previously described (5). The viability of the CBP siRNA, control siRNA, and mock-transfected SH-SY5Y cells were similarly measured by MTT after ionizing radiation exposure. All experiments were carried out in triplicate and the average values and SDs were calculated.

siRNA-mediated silencing of CBP

SH-SY5Y cells were plated at a density of 2×10^6 cells per 10-cm plate 24 hours before transfection. The following day, the cells were transfected either with smart pool CBP siRNA, or the scrambled nontargeting siRNA (Dharmacon Inc.) using nucleofactor kit V (Lonza) according to the manufacturer's instruction. Mock transfection as well as the nontargeting scrambled siRNA transfection served as controls. The knock down of CBP was determined 72 hours post transfection by immunoblotting using anti-CBP antibodies.

Immunoblot analysis

For immunoblot analyses, whole-cell extracts were prepared from human NB cell lines following ionizing radiation treatment. The proteins were separated by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and immunoblotted for γ-H2AX, CBP, Bax, or control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies. The presence of proteins were visualized using Enhanced Chemiluminescence plus.

Fractionation and immunoprecipitation

The SH-SY5Y cells and the CBP-knockdown SH-SY5Y cells were fractionated into cytosolic and nuclear fractions using low-salt and high-salt buffer as previously described (17). The fractions were immunoprecipitated using an anti-acetyl-lysine antibody (Santa Cruz Biotechnology) or K103-acetylated lysine antibody (Cell Signaling) in CHAPS buffer, and complexes were immunoblotted with Ku70 antibody (3).

Clonogenic assay

To determine the survivability of NB cells after various treatments, we used a clonogenic assay (18). Cells were trypsinized and seeded in triplicate into 60-mm dishes (400 cells for unirradiated control and 40,000 for irradiated cells) and allowed to grow undisturbed for 1 week. Colonies were stained with crystal violet, and counted manually using a Leica microscope. The percentage of surviving fraction at different doses was calculated as previous described (18).

Immunofluorescent microscopy and quantification of γ-H2AX foci

NB cells and controls were seeded in chamber slides. At different time points following irradiation at 2 Gy, cells were fixed with 4% paraformaldehyde for 15 minutes, washed with PBS, and permeabilized in 0.2% Triton-X100. After blocking with 5% normal goat serum (NGS) for 1 hour, samples were incubated with anti–phospho-histone γ-H2AX (Ser139, clone JBW301) mouse monoclonal antibody (Millipore) at a 1:500 dilution in 2.5% NGS-PBS overnight at 4°C, followed by incubation with the secondary antibody Alexa Fluor 568 goat anti-mouse-IgG (1:1,000) for 1 hour. Cells were then washed with PBS and mounted using mounting solution with 4′, 6-diamidino-2-phenylindole (DAPI; Invitrogen). Images of γ-H2AX foci and nuclei were taken using Leica DMR fluorescent microscope at ×40. Quantitative analysis of foci was carried out using Image-J as previously described (19). To test for variation between experiments, at least 100 cells from 3 different experiments were scored for each data point. The mean number of foci per cell and the SD from 3 independent measures were calculated.

The comet assay

The neutral comet assay was conducted using a Trevigen Comet Assay Kit (4250-050-K; Trevigen) according to the manufacturer's protocol. SH-SY5Y, SH-EP1, CBP- knocked down SH-SY5Y cells, or control siRNA-treated SH-SY5Y cells were exposed to radiation dose of 10 Gy and subjected to comet assay at the indicated time points of 0, 1, or 3 hours. The comet images were captured using fluorescent microscopy after staining with SYBR green. Average comet tail moment (percentage of DNA in tail length) was scored for 3 fields (>50 comets in each field) using the Comet Score software (TriTek). The results are expressed as mean ± SD.

Results

Ku70 is acetylated in the cytoplasm and in the nucleus after irradiation

We have shown previously that acetylation of cytoplasmic Ku70 triggers Bax release and NB cell apoptosis (5). However, the role of Ku70 acetylation in NB DNA nuclear-repair responses is unknown. In this study we have determined that ionizing radiation induces Ku70 acetylation in SH-SY5Y cells, N-type NB cells (Fig. 1A, left), but not in SH-EP1 cells, S-type NB cells, (Fig. 1A, right). In addition in SH-SY5Y N-type cells, both cytoplasmic and nuclear Ku70 are acetylated 24 hours after 10 Gy ionizing radiation (Fig. 1B). Interestingly, when we determined the level of Ku70 in the cytoplasm and in the nucleus at 0, 2, 4, and 6 hours after ionizing radiation, we found the level of Ku70 is similar in the cytoplasm and in the nucleus of untreated cells (0 hour; Fig. 1C). Two hours after ionizing radiation, however, there is a visible reduction in the cytoplasmic Ku70 level, and simultaneously, nuclear Ku70 level increases. These changes of Ku70 level between the cytoplasm and the nucleus are evident for 6 hours after treatment, suggesting DNA damage induced by ionizing radiation causes Ku70 to translocate from the cytoplasm to the nucleus.

Kinetics of DNA repair in NB cells in response to irradiation

To investigate the DNA-damage response of NB cells to ionizing radiation, we used phosphorylation of histone H2AX (γ-H2AX) as a marker of DNA damage (20). Interestingly, when we compared the kinetics of γ-H2AX expression using immunoblot analyses in N-type versus S-type NB cells, we found that in SH-SY5Y N-type cells the rate of disappearance of γ-H2AX is prolonged up to 8 hours after 10 Gy ionizing radiation treatment (Fig. 2A, left). In contrast, in SH-EP1 S-type cells, γ-H2AX disappears within 2 hours of treatment (Fig. 2A, right). These results suggest that repair of DNA damage is much faster in SH-EP1 cells compared with that of SH-SY5Y cells. Similar results were obtained when we used a lower dose of ionizing radiation, 2 Gy, and counted the total foci of γ-H2AX of over 100 cells for each treatment. The immunocytochemistry staining at 0, 3, and 7 hours after 2 Gy ionizing radiation treatment is shown in Fig. 2B. The compilation of the total γ-H2AX foci counts at various times after

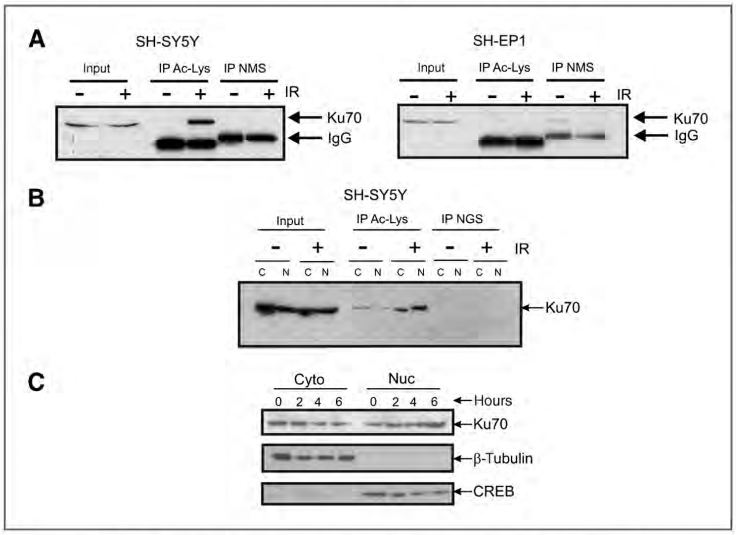

Figure 1. Ionization radiation induces Ku70 acetylation and translocation from cytoplasm to nucleus. A, N-type SH-SY5Y and S-type SH-EP1 cells received 10 Gy radiation and, 24 hours later, lysates were immunoprecipitated using an anti-acetyl-lysine antibody (Cell Signaling) and probed for Ku70. NMS, normal mouse serum. B, SH-SY5Y cells were irradiated at 10 Gy and, 24 hours later, lysates were fractionated into cytoplasmic and nuclear fractions. Both fractions were immunoprecipitated (IP) using an anti-acetyl-lysine antibody (Santa Cruz Biotechnology) and probed for Ku70. NGS, normal goat serum. C, SH-SY5Y cells were irradiated at 10 Gy. The irradiated cells were harvested 2, 4, or 6 hours later. Lysates were fractionated into cytoplasmic and nuclear fractions and then immunoblotted for Ku70, β-tubulin as a cytoplasmic marker, and CREB as a nuclear maker.

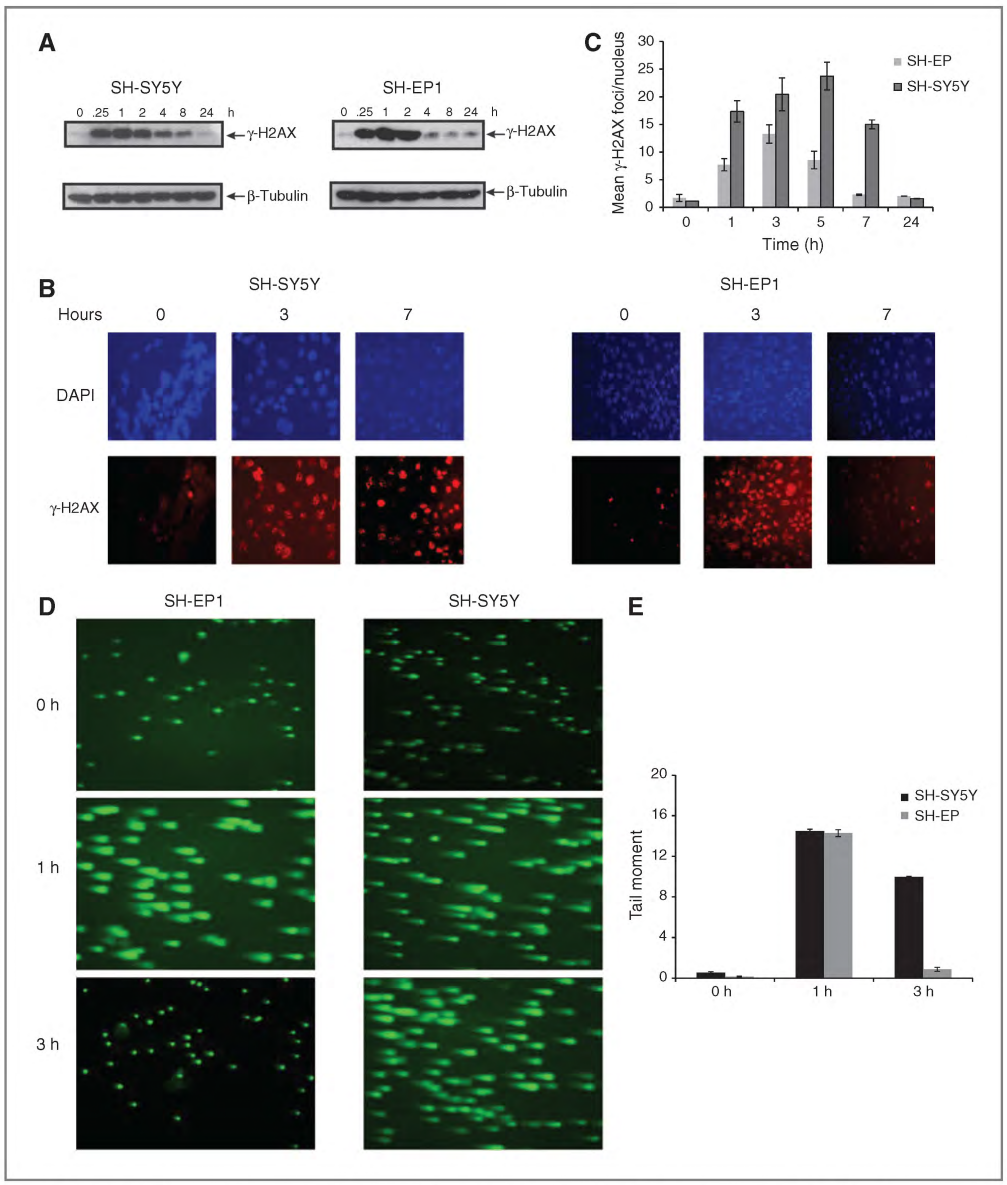

Figure 2. Kinetics of DNA repair in neuroblastoma cells in response to irradiation. A, SH-SY5Y and SH-EP1 cells were irradiated at 10 Gy. At various times as indicted after ionizing radiation, lysates were collected and immunoblotted for γ-H2AX and β-tubulin. B, SH-SY5Y and SH-EP1 cells were irradiated at 2 Gy. At various times as indicated after ionizing radiation, immunofluorescence for γ-H2AX was conducted. Only data for 0, 3, and 7 hours are shown. C, compilation of the number of foci of γ-H2AX per cell after ionizing radiation as shown in B at times indicated. The results are expressed as mean ± SD ($n = 3$, 100 foci per sample). D and E, SH-SY5Y cells and SH-EP1 cells were irradiated at 10 Gy. At 0, 1, and 3 hours later, the cells were subjected to the Comet assay as described. The fluorescent microscopy staining with SYBR green was shown in D. The average comet tail moment (percentage of DNA in tail length) was scored in 3 different fields (at least 50 comets per field) shown in E. The results are expressed as mean ± SD ($n = 3$).

ionizing radiation treatment is shown in Fig. 2C for SH-SY5Y cells and SH-EP1 cells. The results are consistent with the immunoblot data (shown in Fig. 2A) when a higher dose (10 Gy) of ionizing radiation is used in that S-type NB cells have a faster kinetics of repairing DNA than that of the N-type NB cells. Similar results were also obtained when a direct measurement of DNA breaks were checked using a Comet Assay, which measures the DNA breaks at the time when the cells are lysed (21). Results shown in Fig. 2D and E show that SH-EP1 has a faster kinetics of DNA repair (measuring at 1 and 3 hours following ionizing radiation) than that of SH-SY5Y cells after 10 Gy treatment.

When we determined cell viability using MTT assay following ionizing radiation, we found that in N-type NB cells (SH-SY5Y and IMR32 cells) viability decreases proportional to the ionizing radiation dose (5, 10, or 20 Gy) used. In contrast, in SH-EP1 cells, similar to the IMR90, a fibroblast type cell line, the viability dropped minimally following (down to 85% of control) various ionizing radiation treatments (Fig. 3A). Similar results were obtained when a clonogenic assay was used to assess the survivability of SH-SY5Y cells and SH-EP1 cells following various doses of ionizing radiation (Fig. 3B).

The reduction of cell viability after ionizing radiation seen in SH-SY5Y cells, but not in SH-EP1 cells, is consistent with our previous findings showing that cytoplasmic Ku70 is acetylated in SH-SY5Y cells but not in SH-EP1 cells (Fig. 1). When we immunoprecipiated Bax after 10 Gy ionizing radiation in these 2 cell types, we found much less Ku70 is associated with Bax in the SH-SY5Y cells compared with that of SH-EP1 cells. These results indicate that Bax is released from Ku70 after ionizing radiation in SH-SY5Y cells (Fig. 3C, left) but not in SH-EP1 cells (Fig. 3C, right).

Interestingly, when we further studied SH-EP1 cells that survived ionizing radiation treatment, we found that they showed resistance to the effects of cisplatin, a chemotherapeutic agent known to induce cell death in NB cells (Fig. 4A; ref. 22). Furthermore, the SH-EP1 cells had faster growth rates in low serum conditions (0.1 or 0.5%) when compared with unirradiated cells (Fig. 4B).

CBP regulates Ku70 acetylation in response to irradiation in NB cells

We have shown previously that CBP regulates Ku70 acetylation in NB cells. Overexpression of CBP or CBP knock down will alter the response of histone deacetylase

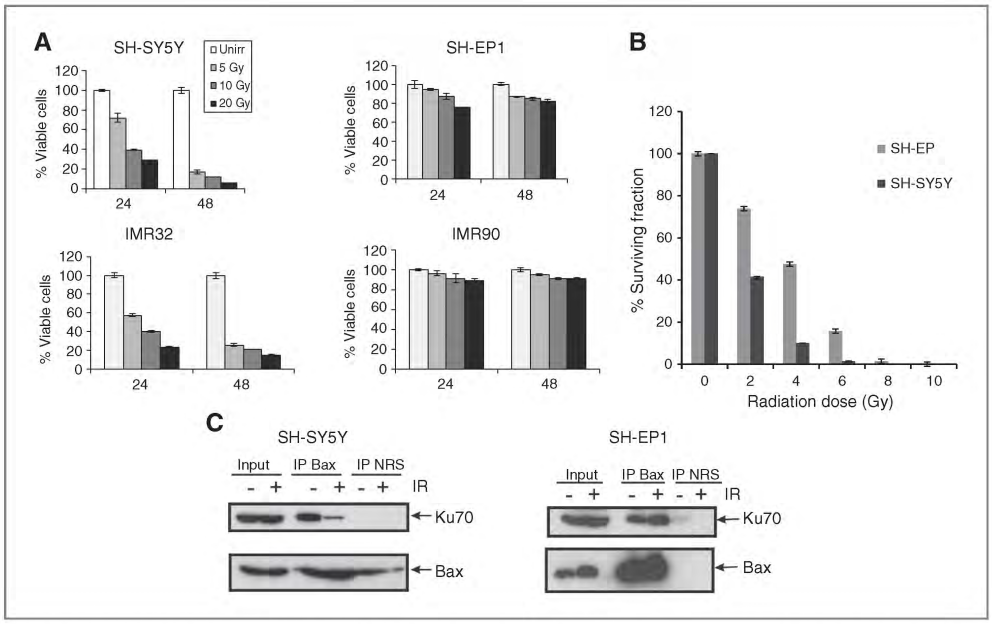

Figure 3. Irradiation reduces neuroblastoma cell viability. A, SH-SY5Y, IMR32, SH-EP1, and IMR90 cells were irradiated at 5, 10, or 20 Gy. Cell viability was determined by MTT assay after 24 or 48 hours. Results are expressed as the percentage of viable cells compared with unirradiated control cells (mean ± SD, $n = 3$). B, SY-SH5Y cells and SH-EP1 cells were irradiated at various doses of ionizing radiation as indicated. Immediately after irradiation, cells were plated. One week after plating, the colonies were fixed with glutaraldehyde (6.0% v/v), stained with crystal violet (0.5%w/v), and counted using a microscope. The results are expressed as the percentage of the number of colonies (mean ± SD, $n = 3$) compared with the number of colonies of unirradiated cells. C, SH-SY5Y cells (left) and SH-EP1 cells (right) were irradiated at 10 Gy. Twenty-four hours later, cell lysates were immunoprecipitated with anti-Bax antibodies, and then immunoblotted for Ku70 or Bax. NRS, normal rabbit serum.

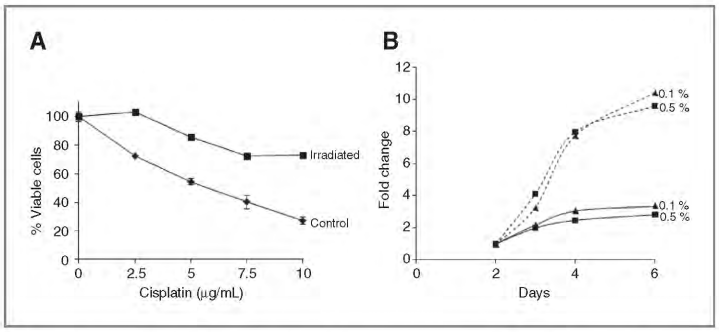

Figure 4. Irradiated SH-EP1 cells are more resistant to cisplatin and grow better in low-serum media. A, SH-EP1 cells are irradiated at 10 Gy. Twenty-four hours later, the unirradiated or irradiated cells were treated with various doses of cisplatin as indicated. Cell viability was determined using sulforhodamine assay 24 hours after drug treatment. Results are expressed as the percentage of viable cells compared with unirradiated control cells (mean ± SD, $n = 3$). B, SH-EP1 cells were irradiated at 10 Gy and, 24 hours later, the cells were plated at serum concentrations of 0.1% and 0.5%. Cell viability was determined at days 2, 3, 4, or 6 using sulforhodamine assay. Results are expressed as the fold of viable cells compared with the day 2 value (equals to 1 fold; mean ± SD, $n = 3$). The solid lines represent control cells and the broken lines show irradiated cells.

inhibitor treatment that induces cell death in NB cells (6). In this current study, we tested whether CBP regulates Ku70 acetylation in response to DNA damage induced by ionizing radiation. Because we did not observe Ku70 acetylation in S-type cells (Fig. 1A) after ionizing radiation, we focused our studies on SH-SY5Y cells.

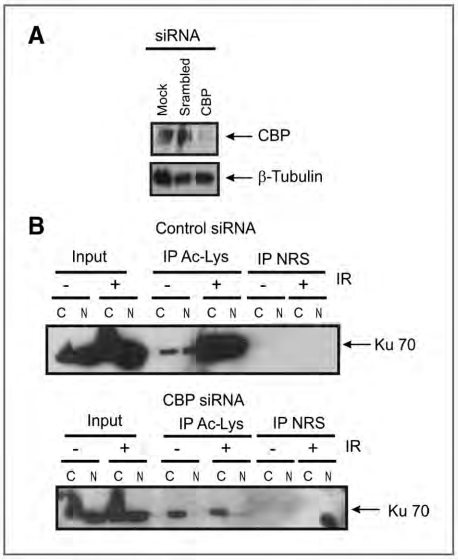

Figure 5. CBP regulates Ku70 acetylation in response to irradiation in NB cells. SH-SY5Y cells were transfected with either control scrambled siRNA or CBP siRNA. Forty-eight hours after transfection, the cells were either collected to determine the depletion of CBP (A) or irradiated at 10 Gy (B). Twenty-four hours following ionizing radiation, lysates were fractionated into cytoplasmic and nuclear fractions. Each fraction was immunoprecipitated using an anti-acetyl-lysine antibody, and immunoblotted for Ku70.

To test whether CBP regulates Ku70 acetylation following ionizing radiation treatment in NB cells, we knocked down CBP using CBP specific siRNA and tested (Fig. 5A) whether Ku70 is acetylated in response to ionizing radiation (10 Gy; Fig. 5A). Twenty four hours after ionizing radiation treatment, scrambled siRNA or CBP-siRNA-transfected cells with and without exposure to ionizing radiation were fractionated to separate the cytoplasmic and nuclear fractions. Samples were then immunoprecipitated using an anti-acetyl-lysine antibody (Santa Cruz Biotechnology) and then immunoblotted for Ku70. As shown in Fig. 5B, CBP knock down reduces the level of Ku70 acetylation in both the cytosolic and nuclear fractions following ionizing radiation.

To determine if there was a change in ionizing radiation-induced DNA-damage-repair activity following CBP knockdown, we treated scrambled siRNA control or CBP-siRNA-transfected SH-SY5Y cells with ionizing radiation (2 Gy), and the kinetics of γ-H2AX was determined by immunocytochemistry using γ-H2AX–specific antibodies (Fig. 6A). The results shown in Fig. 6B are the number of γ-H2AX foci per nucleus (100 nucleus). These results show that the CBP-knockdown cells show a faster kinetics of the disappearance of γ-H2AX compared with the scrambled siRNA control. These results suggest that CBP may be regulating the DNA-damage response, possibly by regulating Ku70 acetylation, induced by ionizing radiation. Similar results were obtained when the Comet Assay was used to determine the total DNA breaks at the time of cell lysis (Figs. 6C and D). A clonogenic assay was also carried out simultaneously to identify the survival ability after ionizing radiation-induced DNA damage when CBP is depleted. Results in Fig. 7A show CBP-knock down SH-SY5Y cells showing significantly higher survival when exposed to various doses of ionizing radiation compared with control-transfected cells. Interestingly,

transfection of a Ku70 mutant which has K539 and K542 converted to arginine (K539/542R) has no effect on ionizing radiation-induced reduction in cell viability in SH-SY5Y cells (Fig. 7B), suggesting that acetylation of K539and K542 may not be responsible in regulating SH-SY5Y cell DNA repair following ionizing radiation treatment.

Discussion

Of the various types of DNA damage, DSBs induced by ionization radiation is most toxic to dividing cells (23). Efficient repair of DNA DSB is critical for maintaining genomic stability and cell viability (24). In mammalian cells, DNA-repair activity in response to ionizing radiation-

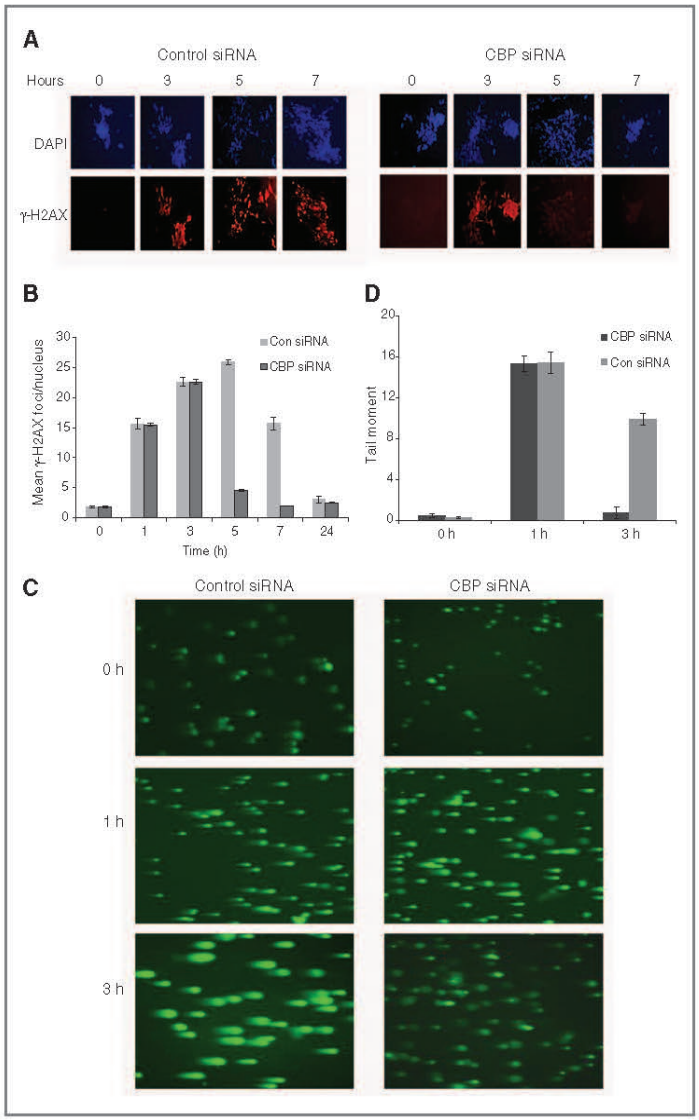

Figure 6. CBP depletion shortens the DNA repair time in neuroblastoma cells. A, SH-SY5Y cells transfected with CBP siRNA or control siRNA were irradiated at 2 Gy. At different times following ionizing radiation, immunofluorescence for γ-H2AX was conducted as described in the Materials and Methods section. Data for 0, 3, 5, and 7 hours after ionizing radiation are shown in A. B, compilation of the number of foci of γ-H2AX per cells after ionizing radiation described in A at times indicated. The results are expressed as mean ± SD ($n = 3$, 100 foci per sample). C and D, SH-SY5Y cells were transfected with CBP siRNA or control siRNA. Two days after transfection, the cells were irradiated at 10 Gy. At 0, 1, and 3 hours after ionizing radiation, the cells were subjected to the Comet assay as described. The fluorescent microscopy staining with SYBR green was shown in C. The average comet tail moment (percentage of DNA in tail length) was scored in 3 different fields (at least 50 comets per field) shown in D. The results are expressed as mean ± SD ($n = 3$).

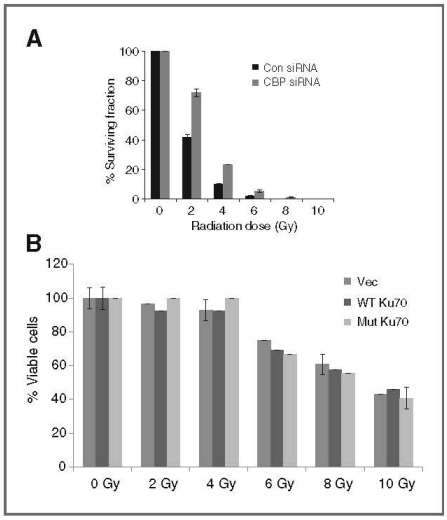

Figure 7. CBP depletion increases the survival of neuroblastoma cells after irradiation. A, SH-SY5Y cells were transfected with control siRNA or CBP-specific siRNA. Two days after transfection, the cells were irradiated at various doses of irradiation. Immediately after irradiation, cells were plated. One week after plating, the colonies were fixed with glutaraldehyde (6.0% v/v), stained with crystal violet (0.5%w/v), and counted using a microscope. The results are expressed as the percentage of the number of colonies (mean ± SD, $n = 3$) compared with the number of colonies of ionizing radiation-treated cells without siRNA transfection. B, SH-SY5Y cells were transfected with a Ku70 K539/K542R expression vector. Two days after transfection, the cells were treated with various doses of irradiation as indicated. One day after irradiation, the cell viability was determined by MTT assay. Results are expressed as the percentage of viable cells compared with unirradiated control cells (mean ± SD, $n = 3$).

induced DSBs occurs via 2 routes: homologous recombination (HR) and NHEJ (25). HR is an accurate form of DNA repair that is restricted to DNA repair occurring at S-phase following DNA replication. In contrast, NHEJ operates throughout the cell cycle and is considered the major pathway for the ionizing radiation-induced DSB DNA repair (26–28).

The Ku70-Ku80 heterodimer plays a key role in the NHEJ DNA-repair pathway. The initial step in the NHEJ pathway is detection of the DSB by Ku heterodimer, followed by recruitment of the catalytic subunit of DNA-dependent protein kinase (DNA-PKc) to form the active DNA-protein kinase-dependent holoenzyme (29). Deficiency of either NHEJ proteins in cells or mutations in Ku70, Ku80, or DNA-PKc leads to ionizing radiation sensitization (23). Previous studies have shown that Ku70 acetylation blocks Ku70 binding to broken-end DSB DNA (16). In this study, we have shown that Ku70 is acetylated upon ionizing radiation not only in the cytosol but also in the nucleus of N-type, but not in S-type, NB cells. We have also shown that in N-type, but not in S-type, NB cells, Ku70 acetylation in the cytosol releases Bax from Ku70 resulting in Bax translocation to the mitochondria causing cell death (5). However, expression of Ku70 K539/542R mutant does not rescue SH-SH5Y cell death induced by ionizing radiation, suggesting that Bax may not regulate cell death triggered by ionizing radiation in this cell type.

Our results have also shown that N-type cells show prolonged repair activity and more apoptotic cell death when compared with the S-type NB cells following ionizing radiation, suggesting that regulation of Ku70 acetylation may play a role in NB cell survival. We have shown that the irradiated SH-EP1 cells show resistance to the chemotherapeutic drug cisplatin, and that they grow better in lower serum conditions. These results suggest that ionizing radiation induces cell death of least survivable SH-EP1 cells, and the remaining SH-EP1 cells, having a faster DNA-repair rate and low Ku70 acetylation following ionizing radiation, adopt a better survival mechanism that is resistant to DNA-damaging compounds such as cisplatin. However, whether this survival mechanism in SH-EP1 cells is related to Ku70 acetylation affecting its DNA binding for repair remains to be tested.

Our results show that CBP acetylates Ku70 in the cytosol and in the nucleus of NB cells; depleting CBP lowers Ku70 acetylation in both compartments. Consistent with our model, the repair activity of irradiated, CBP-depleted SH-SY5Y cells is more robust than control cells, and CBP-depleted cells have higher survivability, judging by the increase of colonies of CBP-depleted cells on ionizing radiation in the clonogenic assay. These results suggest that CBP and deacetylases regulate Ku70 functions in NB cells. However, this mechanism may be cell-type specific. Ogiwara and colleagues have shown that, in human lung cancer H1299 cells, depletion of CBP and p300 results in repressing DNA-repair activity, which is the opposite of what we report here (30). Together, their results as well as ours suggest that the role of CBP in DNA repair activity may be cell-context specific. What regulates this specificity remains to be determined.

Taken together, our data for the first time in NB cells identify Ku70 acetylation in response to ionizing radiation-induced DNA damage to be responsible cell death exposed to ionizing radiation. Our previous study, showing low level CBP expression in S-type cells compared with that in N-type cells, indicates that the resistance of S-type NB cells to radiation and faster DNA-repair activity may be due to low-level CBP expression (6). These results also provide novel insight into the differences between N-type and S-type NB cells in response to ionizing radiation-induced DNA damage. These findings provide a rationale for testing modulators of Ku70 acetylation in clinical trials together with radiotherapy or DNA-damaging agents for the treatment of NB.

Disclosure of Potential Conflicts of Interest

A.W. Opipari Jr. is employed as a co-founder and Senior Director of and has ownership interest (including patents) in Lycer, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: C. Subramanian, A.W. Opipari, Jr., V.P. Castle, R.P.S. Kwok

Development of methodology: C. Subramanian, M. Hada, V.P. Castle, R.P.S. Kwok

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Subramanian, M. Hada, V.P. Castle, R.P.S. Kwok

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Subramanian, M. Hada, A.W. Opipari, Jr., V.P. Castle, R.P.S. Kwok

Writing, review, and/or revision of the manuscript: C. Subramanian, A.W. Opipari, Jr., V.P. Castle, R.P.S. Kwok

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V.P. Castle, R.P.S. Kwok

Study supervision: C. Subramanian, R.P.S. Kwok

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References

1. Featherstone C, Jackson SP. Ku a DNA repair protein with multiple cellular functions? Mutat Res 1999;434:3–15.
2. Hammel M, Cai B, Ye R, Phipps BM, Rambo RP, Hura GL, et al. Ku and DNA-dependent protein kinase dynamic conformations and assembly regulate DNA binding and the initial non-homologous end joining complex. J Biol Chem 2010;285:1414–23.
3. Sawada M, Sun W, Hayes P, Leskov K, Boothman DA, Matsuyama S, et al. Ku70 suppresses the apoptotic translocation of Bax to mitochondria. Nat Cell Biol 2003;5:320–9.
4. Cohen HY, Lavu S, Bitterman KJ, Hekking B, Imahiyerobo TA, Miller C, et al. Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. Mol Cell 2004;13:627–38.
5. Subramanian C, Opipari AW Jr, Bian X, Castle VP, Kwok RP. Ku70 acetylation mediates neuroblastoma cell death induced by histone deacetylase inhibitors. Proc Natl Acad Sci U S A 2005;102:4842–7.
6. Subramanian C, Jarzembowski JA, Opipari AW Jr, Castle VP, Kwok RP. CREB-binding protein is a mediator of neuroblastoma cell death induced by the histone deacetylase inhibitor trichostatin A. Neoplasia 2007;9:495–503.
7. Weterings E, van Gent DC. The mechanism of non-homologous end-joining: a synopsis of synapsis. DNA Repair (Amst) 2004;3:1425–35.
8. Wang J, Dong X, Reeves WH. A model for Ku heterodimer assembly and interaction with DNA. Implications for the function of Ku antigen. J Biol Chem 1998;273:31068–74.
9. Wu X, Lieber MR. Protein–protein and protein–DNA interaction regions within the DNA end-binding protein Ku70-Ku86. Mol Cell Biol 1996;16:5186–93.
10. Vandersickel V, Mancini M, Slabbert J, Marras E, Thierens H, Perletti G, et al. The radiosensitizing effect of Ku70/80 knockdown in MCF10A cells irradiated with X-rays and p(66)+Be(40) neutrons. Radiat Oncol 2010;5:30.
11. Gu Y, Jin S, Gao Y, Weaver DT, Alt FW. Ku70-deficient embryonic stem cells have increased ionizing radiosensitivity, defective DNA end-binding activity, and inability to support V(D)J recombination. Proc Natl Acad Sci U S A 1997;94:8076–81.
12. Nussenzweig A, Chen C, da Costa Soares V, Sanchez M, Sokol K, Nussenzweig MC, et al. Requirement for Ku80 in growth and immunoglobulin V(D)J recombination. Nature 1996;382:551–5.
13. Sawada M, Hayes P, Matsuyama S. Cytoprotective membrane-permeable peptides designed from the Bax-binding domain of Ku70. Nat Cell Biol 2003;5:352–7.
14. Nothwehr SF, Martinou JC. A retention factor keeps death at bay. Nat Cell Biol 2003;5:281–3.
15. Walker JR, Corpina RA, Goldberg J. Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. Nature 2001;412:607–14.
16. Chen CS, Wang YC, Yang HC, Huang PH, Kulp SK, Yang CC, et al. Histone deacetylase inhibitors sensitize prostate cancer cells to agents that produce DNA double-strand breaks by targeting Ku70 acetylation. Cancer Res 2007;67:5318–27.
17. Subramanian C, Jarzembowski JA, Opipari AW Jr, Castle VP, Kwok RP. HDAC6 deacetylates Ku70 and regulates Ku70-Bax binding in neuroblastoma. Neoplasia 2011;13:726–34.
18. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells *in vitro*. Nat Protoc 2006;1:2315–9.
19. Cai Z, Vallis KA, Reilly RM. Computational analysis of the number, area and density of gamma-H2AX foci in breast cancer cells exposed to (111)In-DTPA-hEGF or gamma-rays using Image-J software. Int J Radiat Biol 2009;85:262–71.
20. Taneja N, Davis M, Choy JS, Beckett MA, Singh R, Kron SJ, et al. Histone H2AX phosphorylation as a predictor of radiosensitivity and target for radiotherapy. J Biol Chem 2004;279:2273–80.
21. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988;175:184–91.
22. Bian X, Giordano TD, Lin HJ, Solomon G, Castle VP, Opipari AW, et al. Chemotherapy-induced apoptosis of S-type neuroblastoma cells requires caspase-9 and is augmented by CD95/Fas stimulation. J Biol Chem 2004;279:4663–9.
23. Mahaney BL, Meek K, Lees-Miller SP. Repair of ionizing radiation-induced DNA double-strand breaks by non-homologous end-joining. Biochem J 2009;417:639–50.
24. Hakem R. DNA-damage repair; the good, the bad, and the ugly. EMBO J 2008;27:589–605.
25. Cahill D, Connor B, Carney JP. Mechanisms of eukaryotic DNA double strand break repair. Front Biosci 2006;11:1958–76.
26. Lobrich M, Jeggo PA. The impact of a negligent G2/M checkpoint on genomic instability and cancer induction. Nat Rev Cancer 2007;7:861–9.
27. Helleday T, Lo J, van Gent DC, Engelward BP. DNA double-strand break repair: from mechanistic understanding to cancer treatment. DNA Repair (Amst) 2007;6:923–35.
28. O'Driscoll M, Jeggo PA. The role of double-strand break repair - insights from human genetics. Nat Rev Genet 2006;7:45–54.
29. Weterings E, Chen DJ. The endless tale of non-homologous end-joining. Cell Res 2008;18:114–24.
30. Ogiwara H, Ui A, Otsuka A, Satoh H, Yokomi I, Nakajima S, et al. Histone acetylation by CBP and p300 at double-strand break sites facilitates SWI/SNF chromatin remodeling and the recruitment of non-homologous end joining factors. Oncogene 2011;30:2135–46.

Appendix B: A copy of a peer-reviewed review article published in the Journal of Cell Death in 2014 (Vol 7, page 11-13) entitled "Regulation of Ku70-Bax complex in cells" is included. I was the first name author of the article.

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Journal of Cell Death

Regulation of Ku70-Bax Complex in Cells

Manila Hada and Roland P.S. Kwok

Departments of Obstetrics and Gynecology, and Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI, USA.

ABSTRACT: Ku70, a DNA repair factor in the nucleus, also regulates cell death by binding to the apoptotic protein Bax in the cytoplasm. Acetylation of Ku70 triggers Bax release resulting in Bax dependent cell death. Thus dissociating Bax from Ku70, either by inhibiting histone deacetylase 6 (HDAC6) that deacetylates Ku70 or by increasing Ku70 acetylation induces cell death. Our results showed that in neuroblastoma cells, the depletion of Ku70 results in Bax-dependent cell death. This model provides a rationale for screening Ku70 acetylation modulators that can be tested in clinical trials, either alone or in combination with radiotherapy or DNA-damaging agents for the treatment of cancer.

KEYWORDS: Ku70, Bax, HDAC6, CBP, apoptosis

CITATION: Hada and Kwok. Regulation of Ku70-Bax Complex in Cells. *Journal of Cell Death* 2014:7 11–13 doi:10.4137/JCD.S13695.

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COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

CORRESPONDENCE: rkwok@umich.edu

Introduction

Ku70 was originally discovered as an auto-antigen and later characterized as a DNA binding component of the non-homologous end joining (NHEJ) double strand break (DSB) repair.[1] Ku70 dimerizes with Ku80, and the Ku70–Ku80 complex binds to DSB DNA. The Ku70/80 heterodimer then recruits DNA dependent protein kinase catalytic subunit (DNA-PKcs) to the DSB, followed by auto-phosphorylation of the DNA-PKcs and other DNA repair proteins, including Ku70 and XRCC4.[2] Over the last several years, our laboratory has investigated Ku70's cytoplasmic function, the regulation of cell death through interaction with an apoptotic protein, Bax. Here, we will describe our model and discuss the implications of this model.

Model of Ku70 and Implications

Although Ku70 was originally found in the nucleus, it was found to bind to Bax in the cytoplasm.[3–5] The Bax-binding domain of Ku70 was mapped to the residues between residues 578–609 of Ku70.[6] A five-residue peptide corresponding to the Ku70 596–600 has been demonstrated to bind to Bax and block Bax-mediated cell death.[7] A study by Cohen et al has mapped the acetylation sites of Ku70.[8] When two of these sites, K539 and K542, are acetylated, Ku70 dissociates from Bax. However, whether dissociated Bax will induce cell death is not clear as when the HEK293 and HeLa cells were treated with class 1 and class 2 histone deacetylase inhibitors (HDACI) to increase Ku70 acetylation, the cells did not die.[8] Thus, how Ku70-Bax regulated cell death is uncertain.

Using neuroblastoma (NB) cells as model, we have demonstrated a key role for Ku70 in regulating cell death. In NB cells, especially the neuroblastic type (N-type) cells, Ku70 binds to Bax in an acetylation-sensitive manner.[3] Upon the inhibition of HDAC activity, Ku70 is acetylated. Acetylated Ku70 releases Bax allowing it to translocate to mitochondria and trigger cytochrome c release, resulting in caspase-dependent death. Importantly, depleting Ku70 in NB cells triggers cell death, but the cell killing can be stopped by simultaneous depletion of Bax, suggesting that leaving Bax unbound when Ku70 is absent will lead to cell death.[4] Our findings are contrary to the findings in HEK293 and HeLa cells.[8] Our results suggest that in addition to its role in NHEJ

repair, Ku70 may act as a survival factor, at least in NB cells, to block Bax-triggered cell death.[9] These results indicate that Ku70 is a regulatory factor for Bax activity, and that this interaction may be therapeutically targeted in NB cells.

To investigate how the Ku70–Bax complex is regulated by acetylation, we and others have shown that the cAMP-response-element binding protein (CREB)-binding protein (CBP), a transcriptional co-activator and an acetyltransferase, acetylates Ku70 in NB cells.[4,8] CBP depletion causes the down regulation of Ku70 acetylation, resulting in increased resistance to HDAC inhibitor induced cell death. Mutation of K539/K542 of Ku70 to arginine also blocks HDAC inhibitor-induced cell death and blocks Bax release following HDACI treatment, suggesting that K539 and K542 play an important role in regulating Bax binding. In addition to K539 and K542, Ku70 is also acetylated at K282, K317, K331, K338, K544, K553, and K556.[8] Two of these acetylation sites (K282, K317) lie within the Ku70 DNA binding domain. Previous studies have suggested that the acetylation of these lysines in Ku70 down-regulates its DNA binding activity.[10]

Five of the acetylable lysines (K539, K542, K544, K553, and K556) of Ku70 are found within the nuclear localization signal (NLS). As the acetylation of lysine residues within the NLS may regulate nuclear translocation,[11] the acetylation of lysine residues within the NLS of Ku70 may thus also regulate Ku70 nuclear translocation. Thus, it is possible that the acetylation of cytoplasmic Ku70 results in Bax release. Acetylated Ku70 may then be free from Bax and translocate into the nucleus. Our recent results, in fact, have demonstrated that following ionization radiation, both cytoplasmic and nuclear Ku70 are acetylated.[1] Interestingly, cytoplasmic Ku70 was redistributed to the nucleus following irradiation. However, the role of Ku70 that translocates into the nucleus is still not clear.

We have shown that depleting CBP in NB cells reduces Ku70 acetylation and enhances DNA repair activity, suggesting that Ku70 acetylation may have an inhibitory role in DNA repair.[12] Several studies, however, have demonstrated that the level of Ku70 correlates with radiosensitivity.[13] Ku70 knock down increases the sensitivity of radiation treatment in human cancer cell lines[14] while increasing Ku70 levels in cells reduces radiosensitivity. It has been proposed that the altered sensitivity to irradiation is because of changes in Ku70 levels that may be the result of Ku70 mediated DNA repair following irradiation. However, our results provide an additional rationale for testing modulators of Ku70 acetylation in treatment with radiotherapy or DNA-damaging agents for the treatment of NB.

As lysine acetylation is regulated by both acetyltransferases and deacetylases, we sought to identify the deacetylase that deacetylates Ku70 in the cytoplasm. A previous study has shown that a class III HDAC, SIRT3, was able to deacetylate Ku70,[15] but it is uncertain how SIRT3, which is mainly localized in the mitochondria, is involved in regulating Ku70 acetylation resulting in Bax dissociation. In our studies, we used class specific HDAC inhibitors to inhibit HDAC activity in cells. We have shown that a HDAC6 specific inhibitor, tubacin, induces cell death in NB cells.[4] HDAC6 is a class IIb HDAC containing two catalytic domains.[16] HDAC6 is mainly localized in the cytoplasm and has been associated with many cell functions including tubulin stabilization, cell motility, and regulation of the binding between Hsp90 and its cochaperone.[17] In NB cells, HDAC6 forms a complex with Ku70 and Bax, and that depleting HDAC6 has a similar effect as that of tubacin treatment. Furthermore, depleting HDAC6 also increases Ku70 acetylation, releasing Bax from Ku70, causing cell death.[18] Thus, our results show the feasibility of targeting a single HDAC to therapeutically target the Ku70-Bax complex.

The two established functions of Ku70, one in the nucleus (to repair DNA) and one in the cytoplasm (to block Bax activity) are to protect the cell from dying (Fig. 1). However, following an apoptotic stimulation (radiation or DNA-damaging agent treatment), while nuclear Ku70 may still protect cells from dying (discussed below) by repairing DSB DNA, cytoplasmic Ku70 will become an apoptotic protein by releasing Bax following Ku70 acetylation. Whether these two functions of Ku70 following apoptotic stimulations are related or will affect each other is currently not clear. Furthermore, we have shown that following radiation, both cytoplasmic and nuclear Ku70 are acetylated.[1] The cytoplasmic Ku70 was observed to translocate into the nucleus following radiation. Thus, if the Ku70 that enters the nucleus is still acetylated after entering the nucleus, these acetylated Ku70 will have a reduced binding activity for DSB DNA, resulting in lower DNA repair and more cell death. This model predicts that following apoptotic stimuli, Ku70 changes from an anti-apoptotic factor into a pro-apoptotic factor. Currently it is not known to what extent that cytoplasmic Ku70 affects the DNA-repair function of Ku70 in the nucleus. Is it possible that nuclear translocation of Ku70 following radiation depends on the degree of DNA damage caused by radiation, such that larger DNA damage will allow cytoplasmic, acetylated Ku70 to translocate into the nucleus, reducing the DNA-repair activity and allowing the cell to die? If this model is correct, how is it regulated? Currently, we have no answers for these questions.

Conclusions

Our results established a role of Ku70 in regulating cell death by suppressing Bax activity. Dissociating Bax from Ku70, either by pharmacological means, such as HDAC6 specific inhibitors, or by agents that block the interaction between Ku70 and Bax, will result in cell death, at least in NB cells. Our recent results have shown that this model may be specific for NB cells as Ku70 depletion in other cell types does not induce cell death, but sensitizes the cells to radiation or DNA damaging agents (manuscript submitted). Thus, this model provides a rationale for screening small molecules that enhance or block Ku70–Bax binding, or modulate Ku70 acetylation. Agents that block Ku70–Bax binding or increase

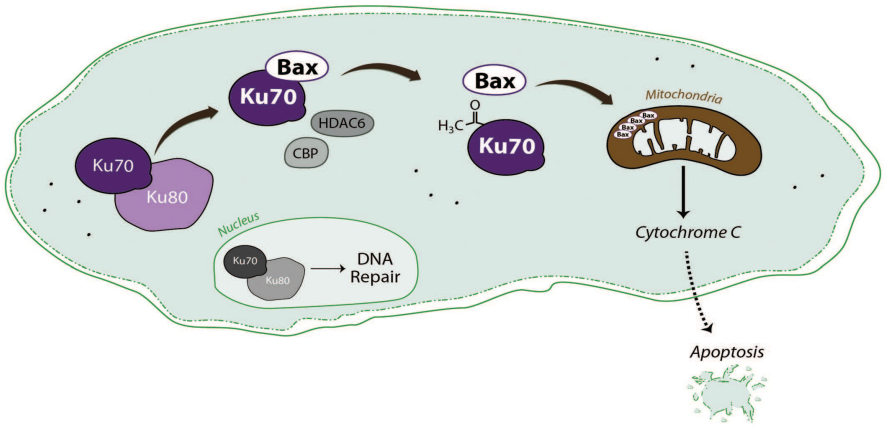

Figure 1. A model for the regulation of Ku70-Bax complex in cells.

Ku70 acetylation may be tested in clinical trials either alone or in combination with radiotherapy or DNA-damaging agents for the treatment of cancer.

Author Contributions

Conceived and designed the experiments: MH, RK. Analyzed the data: MH, RK. Wrote the first draft of the manuscript: RK. Contributed to the writing of the manuscript: MH, RK. Agree with manuscript results and conclusions: MH, RK. Jointly developed the structure and arguments for the paper: MH, RK. Made critical revisions and approved final version: MH, RK. All authors reviewed and approved of the final manuscript.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

REFERENCES

1. Hurwitz JL, et al. Vorinostat/SAHA-induced apoptosis in malignant mesothelioma is FLIP/caspase 8-dependent and HR23B-independent. *Eur J Cancer.* 2012;48(7):1096–1107.
2. Morio T, Kim H. Ku, Artemis, and ataxia-telangiectasia-mutated: signalling networks in DNA damage. *Int J Biochem Cell Biol.* 2008;40(4):598–603.
3. Subramanian C, et al. Ku70 acetylation mediates neuroblastoma cell death induced by histone deacetylase inhibitors. *Proc Natl Acad Sci U S A.* 2005;102(13):4842–4847.
4. Kerr E, et al. Identification of an acetylation-dependant Ku70/FLIP complex that regulates FLIP expression and HDAC inhibitor-induced apoptosis. *Cell Death Differ.* 2012;19(8):1317–1327.
5. Zou H, Volonte D, Galbiati F. Interaction of caveolin-1 with Ku70 inhibits Bax-mediated apoptosis. *PLoS One.* 2012;7(6):e39379.
6. Yoshida T, et al. Bax-inhibiting peptide derived from mouse and rat Ku70. *Biochem Biophys Res Commun.* 2004;321(4):961–966.
7. Gomez JA, et al. Bax-inhibiting peptides derived from Ku70 and cell-penetrating pentapeptides. *Biochem Soc Trans.* 2007;35(pt 4):797–801.
8. Cohen HY, et al. Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. *Mol Cell.* 2004;13(5):627–638.
9. Subramanian C, et al. CREB-binding protein is a mediator of neuroblastoma cell death induced by the histone deacetylase inhibitor trichostatin A. *Neoplasia.* 2007;9(6):495–503.
10. Chen CS, et al. Histone deacetylase inhibitors sensitize prostate cancer cells to agents that produce DNA double-strand breaks by targeting Ku70 acetylation. *Cancer Res.* 2007;67(11):5318–5327.
11. Spilianakis C, Papamatheakis J, Kretsovali A. Acetylation by PCAF enhances CIITA nuclear accumulation and transactivation of major histocompatibility complex class II genes. *Mol Cell Biol.* 2000;20(22):8489–8498.
12. Subramanian C, et al. CREB-binding protein regulates Ku70 acetylation in response to ionization radiation in neuroblastoma. *Mol Cancer Res.* 2013;11(2):173–181.
13. Gu Y, et al. Ku70-deficient embryonic stem cells have increased ionizing radiosensitivity, defective DNA end-binding activity, and inability to support V(D)J recombination. *Proc Natl Acad Sci U S A.* 1997;94(15):8076–8081.
14. Vandersickel V, et al. The radiosensitizing effect of Ku70/80 knockdown in MCF10A cells irradiated with X-rays and p(66) + Be(40) neutrons. *Radiat Oncol.* 2010;5:30.
15. Sundaresan NR, et al. SIRT3 is a stress-responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku70. *Mol Cell Biol.* 2008;28(20):6384–6401.
16. Grozinger CM, Hassig CA, Schreiber SL. Three proteins define a class of human histone deacetylases related to yeast Hda1p. *Proc Natl Acad Sci U S A.* 1999;96(9):4868–4873.
17. Lee YS, et al. The cytoplasmic deacetylase HDAC6 is required for efficient oncogenic tumorigenesis. *Cancer Res.* 2008;68(18):7561–7569.
18. Subramanian C, et al. HDAC6 deacetylates Ku70 and regulates Ku70-Bax binding in neuroblastoma. *Neoplasia.* 2011;13(8):726–734.

References

1. Sawada, M., et al., *Ku70 suppresses the apoptotic translocation of Bax to mitochondria.* Nat Cell Biol, 2003. **5**(4): p. 320-9.
2. Elmore, S., *Apoptosis: a review of programmed cell death.* Toxicol Pathol, 2007. **35**(4): p. 495-516.
3. Friedlander, R.M., *Apoptosis and caspases in neurodegenerative diseases.* N Engl J Med, 2003. **348**(14): p. 1365-75.
4. Taylor, R.C., S.P. Cullen, and S.J. Martin, *Apoptosis: controlled demolition at the cellular level.* Nat Rev Mol Cell Biol, 2008. **9**(3): p. 231-41.
5. Czabotar, P.E., et al., *Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy.* Nat Rev Mol Cell Biol, 2014. **15**(1): p. 49-63.
6. Gautier, F., et al., *Bax activation by engagement with, then release from, the BH3 binding site of Bcl-xL.* Mol Cell Biol, 2011. **31**(4): p. 832-44.
7. Billen, L.P., et al., *Bcl-XL inhibits membrane permeabilization by competing with Bax.* PLoS Biol, 2008. **6**(6): p. e147.
8. Lovell, J.F., et al., *Membrane binding by tBid initiates an ordered series of events culminating in membrane permeabilization by Bax.* Cell, 2008. **135**(6): p. 1074-84.
9. Eskes, R., et al., *Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane.* Mol Cell Biol, 2000. **20**(3): p. 929-35.
10. Terrones, O., et al., *Lipidic pore formation by the concerted action of proapoptotic BAX and tBID.* J Biol Chem, 2004. **279**(29): p. 30081-91.
11. Shamas-Din, A., et al., *tBid undergoes multiple conformational changes at the membrane required for Bax activation.* J Biol Chem, 2013. **288**(30): p. 22111-27.
12. Hotchkiss, R.S., et al., *Cell death.* N Engl J Med, 2009. **361**(16): p. 1570-83.
13. Tait, S.W. and D.R. Green, *Mitochondria and cell death: outer membrane permeabilization and beyond.* Nat Rev Mol Cell Biol, 2010. **11**(9): p. 621-32.
14. Hardwick, J.M. and L. Soane, *Multiple functions of BCL-2 family proteins.* Cold Spring Harb Perspect Biol, 2013. **5**(2).
15. Youle, R.J. and A. Strasser, *The BCL-2 protein family: opposing activities that mediate cell death.* Nat Rev Mol Cell Biol, 2008. **9**(1): p. 47-59.
16. Delbridge, A.R. and A. Strasser, *The BCL-2 protein family, BH3-mimetics and cancer therapy.* Cell Death Differ, 2015. **22**(7): p. 1071-80.
17. Lessene, G., P.E. Czabotar, and P.M. Colman, *BCL-2 family antagonists for cancer therapy.* Nat Rev Drug Discov, 2008. **7**(12): p. 989-1000.
18. Davids, M.S. and A. Letai, *Targeting the B-cell lymphoma/leukemia 2 family in cancer.* J Clin Oncol, 2012. **30**(25): p. 3127-35.
19. Kim, H., et al., *Stepwise activation of BAX and BAK by tBID, BIM, and PUMA initiates mitochondrial apoptosis.* Mol Cell, 2009. **36**(3): p. 487-99.
20. Wei, M.C., et al., *tBID, a membrane-targeted death ligand, oligomerizes BAK to release cytochrome c.* Genes Dev, 2000. **14**(16): p. 2060-71.
21. Doerflinger, M., J.A. Glab, and H. Puthalakath, *BH3-only proteins: a 20-year stock-take.* FEBS J, 2015. **282**(6): p. 1006-16.
22. Ren, D., et al., *BID, BIM, and PUMA are essential for activation of the BAX- and BAK-dependent cell death program.* Science, 2010. **330**(6009): p. 1390-3.

23. Gavathiotis, E., et al., *BH3-triggered structural reorganization drives the activation of proapoptotic BAX.* Mol Cell, 2010. **40**(3): p. 481-92.
24. Portt, L., et al., *Anti-apoptosis and cell survival: a review.* Biochim Biophys Acta, 2011. **1813**(1): p. 238-59.
25. Westphal, D., et al., *Molecular biology of Bax and Bak activation and action.* Biochim Biophys Acta, 2011. **1813**(4): p. 521-31.
26. Wolter, K.G., et al., *Movement of Bax from the cytosol to mitochondria during apoptosis.* J Cell Biol, 1997. **139**(5): p. 1281-92.
27. Walensky, L.D. and E. Gavathiotis, *BAX unleashed: the biochemical transformation of an inactive cytosolic monomer into a toxic mitochondrial pore.* Trends Biochem Sci, 2011. **36**(12): p. 642-52.
28. Renault, T.T. and S. Manon, *Bax: Addressed to kill.* Biochimie, 2011. **93**(9): p. 1379-91.
29. Ghibelli, L. and M. Diederich, *Multistep and multitask Bax activation.* Mitochondrion, 2010. **10**(6): p. 604-13.
30. Akgul, C., D.A. Moulding, and S.W. Edwards, *Alternative splicing of Bcl-2-related genes: functional consequences and potential therapeutic applications.* Cell Mol Life Sci, 2004. **61**(17): p. 2189-99.
31. Oltvai, Z.N., C.L. Milliman, and S.J. Korsmeyer, *Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death.* Cell, 1993. **74**(4): p. 609-19.
32. Upton, J.P., et al., *The N-terminal conformation of Bax regulates cell commitment to apoptosis.* Cell Death Differ, 2007. **14**(5): p. 932-42.
33. Antonsson, B., et al., *Inhibition of Bax channel-forming activity by Bcl-2.* Science, 1997. **277**(5324): p. 370-2.
34. Nechushtan, A., et al., *Conformation of the Bax C-terminus regulates subcellular location and cell death.* EMBO J, 1999. **18**(9): p. 2330-41.
35. Garcia-Saez, A.J., et al., *Membrane-insertion fragments of Bcl-xL, Bax, and Bid.* Biochemistry, 2004. **43**(34): p. 10930-43.
36. George, N.M., et al., *Bax contains two functional mitochondrial targeting sequences and translocates to mitochondria in a conformational change- and homo-oligomerization-driven process.* J Biol Chem, 2010. **285**(2): p. 1384-92.
37. Cartron, P.F., et al., *Distinct domains control the addressing and the insertion of Bax into mitochondria.* J Biol Chem, 2005. **280**(11): p. 10587-98.
38. Hsu, Y.T. and R.J. Youle, *Bax in murine thymus is a soluble monomeric protein that displays differential detergent-induced conformations.* J Biol Chem, 1998. **273**(17): p. 10777-83.
39. Westphal, D., R.M. Kluck, and G. Dewson, *Building blocks of the apoptotic pore: how Bax and Bak are activated and oligomerize during apoptosis.* Cell Death Differ, 2014. **21**(2): p. 196-205.
40. Dewson, G. and R.M. Kluck, *Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis.* J Cell Sci, 2009. **122**(Pt 16): p. 2801-8.
41. Chipuk, J.E. and D.R. Green, *How do BCL-2 proteins induce mitochondrial outer membrane permeabilization?* Trends Cell Biol, 2008. **18**(4): p. 157-64.
42. Leber, B., J. Lin, and D.W. Andrews, *Still embedded together binding to membranes regulates Bcl-2 protein interactions.* Oncogene, 2010. **29**(38): p. 5221-30.
43. Guo, B., et al., *Humanin peptide suppresses apoptosis by interfering with Bax activation.* Nature, 2003. **423**(6938): p. 456-61.
44. Nomura, M., et al., *14-3-3 interacts directly with and negatively regulates pro-apoptotic Bax.* J Biol Chem, 2015. **290**(11): p. 6753.

45. Kerr, E., et al., *Identification of an acetylation-dependant Ku70/FLIP complex that regulates FLIP expression and HDAC inhibitor-induced apoptosis.* Cell Death Differ, 2012. **19**(8): p. 1317-27.
46. Zou, H., D. Volonte, and F. Galbiati, *Interaction of caveolin-1 with Ku70 inhibits Bax-mediated apoptosis.* PLoS One, 2012. **7**(6): p. e39379.
47. Wang, Q., et al., *Mono- or double-site phosphorylation distinctly regulates the proapoptotic function of Bax.* PLoS One, 2010. **5**(10): p. e13393.
48. Johnson, B.N., et al., *The ubiquitin E3 ligase parkin regulates the proapoptotic function of Bax.* Proc Natl Acad Sci U S A, 2012. **109**(16): p. 6283-8.
49. Yamaguchi, H. and H.G. Wang, *The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change.* Oncogene, 2001. **20**(53): p. 7779-86.
50. Gardai, S.J., et al., *Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils.* J Biol Chem, 2004. **279**(20): p. 21085-95.
51. Xin, M. and X. Deng, *Protein phosphatase 2A enhances the proapoptotic function of Bax through dephosphorylation.* J Biol Chem, 2006. **281**(27): p. 18859-67.
52. Mimori, T., et al., *Characterization of a high molecular weight acidic nuclear protein recognized by autoantibodies in sera from patients with polymyositis-scleroderma overlap.* J Clin Invest, 1981. **68**(3): p. 611-20.
53. Mimori, T., *Clinical significance of anti-Ku autoantibodies--a serologic marker of overlap syndrome?* Intern Med, 2002. **41**(12): p. 1096-8.
54. de Vries, E., et al., *HeLa nuclear protein recognizing DNA termini and translocating on DNA forming a regular DNA-multimeric protein complex.* J Mol Biol, 1989. **208**(1): p. 65-78.
55. Boulton, S.J. and S.P. Jackson, *Saccharomyces cerevisiae Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways.* EMBO J, 1996. **15**(18): p. 5093-103.
56. Chen, S., et al., *Accurate in vitro end joining of a DNA double strand break with partially cohesive 3'-overhangs and 3'-phosphoglycolate termini: effect of Ku on repair fidelity.* J Biol Chem, 2001. **276**(26): p. 24323-30.
57. Difilippantonio, M.J., et al., *DNA repair protein Ku80 suppresses chromosomal aberrations and malignant transformation.* Nature, 2000. **404**(6777): p. 510-4.
58. Gu, Y., et al., *Ku70-deficient embryonic stem cells have increased ionizing radiosensitivity, defective DNA end-binding activity, and inability to support V(D)J recombination.* Proc Natl Acad Sci U S A, 1997. **94**(15): p. 8076-81.
59. Singleton, B.K., et al., *Molecular and biochemical characterization of xrs mutants defective in Ku80.* Mol Cell Biol, 1997. **17**(3): p. 1264-73.
60. Errami, A., et al., *Ku86 defines the genetic defect and restores X-ray resistance and V(D)J recombination to complementation group 5 hamster cell mutants.* Mol Cell Biol, 1996. **16**(4): p. 1519-26.
61. Doherty, A.J., S.P. Jackson, and G.R. Weller, *Identification of bacterial homologues of the Ku DNA repair proteins.* FEBS Lett, 2001. **500**(3): p. 186-8.
62. Downs, J.A. and S.P. Jackson, *A means to a DNA end: the many roles of Ku.* Nat Rev Mol Cell Biol, 2004. **5**(5): p. 367-78.
63. Walker, J.R., R.A. Corpina, and J. Goldberg, *Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair.* Nature, 2001. **412**(6847): p. 607-14.
64. Rivera-Calzada, A., et al., *Structural model of full-length human Ku70-Ku80 heterodimer and its recognition of DNA and DNA-PKcs.* EMBO Rep, 2007. **8**(1): p. 56-62.
65. Grundy, G.J., et al., *One ring to bring them all--the role of Ku in mammalian non-homologous end joining.* DNA Repair (Amst), 2014. **17**: p. 30-8.

66. Hu, S., J.M. Pluth, and F.A. Cucinotta, *Putative binding modes of Ku70-SAP domain with double strand DNA: a molecular modeling study.* J Mol Model, 2012. **18**(5): p. 2163-74.
67. Aravind, L. and E.V. Koonin, *SAP - a putative DNA-binding motif involved in chromosomal organization.* Trends Biochem Sci, 2000. **25**(3): p. 112-4.
68. Wang, J., X. Dong, and W.H. Reeves, *A model for Ku heterodimer assembly and interaction with DNA. Implications for the function of Ku antigen.* J Biol Chem, 1998. **273**(47): p. 31068-74.
69. Fell, V.L. and C. Schild-Poulter, *The Ku heterodimer: function in DNA repair and beyond.* Mutat Res Rev Mutat Res, 2015. **763**: p. 15-29.
70. Reaper, P.M., F. di Fagagna, and S.P. Jackson, *Activation of the DNA damage response by telomere attrition: a passage to cellular senescence.* Cell Cycle, 2004. **3**(5): p. 543-6.
71. Shammas, M.A., *Telomeres, lifestyle, cancer, and aging.* Curr Opin Clin Nutr Metab Care, 2011. **14**(1): p. 28-34.
72. Stellwagen, A.E., et al., *Ku interacts with telomerase RNA to promote telomere addition at native and broken chromosome ends.* Genes Dev, 2003. **17**(19): p. 2384-95.
73. Fisher, T.S., A.K. Taggart, and V.A. Zakian, *Cell cycle-dependent regulation of yeast telomerase by Ku.* Nat Struct Mol Biol, 2004. **11**(12): p. 1198-205.
74. Sfeir, A. and T. de Lange, *Removal of shelterin reveals the telomere end-protection problem.* Science, 2012. **336**(6081): p. 593-7.
75. Celli, G.B., E.L. Denchi, and T. de Lange, *Ku70 stimulates fusion of dysfunctional telomeres yet protects chromosome ends from homologous recombination.* Nat Cell Biol, 2006. **8**(8): p. 885-90.
76. Ouyang, H., et al., *Ku70 is required for DNA repair but not for T cell antigen receptor gene recombination In vivo.* J Exp Med, 1997. **186**(6): p. 921-9.
77. Manis, J.P., et al., *Ku70 is required for late B cell development and immunoglobulin heavy chain class switching.* J Exp Med, 1998. **187**(12): p. 2081-9.
78. Gu, Y., et al., *Growth retardation and leaky SCID phenotype of Ku70-deficient mice.* Immunity, 1997. **7**(5): p. 653-65.
79. Li, H., et al., *Deletion of Ku70, Ku80, or both causes early aging without substantially increased cancer.* Mol Cell Biol, 2007. **27**(23): p. 8205-14.
80. Holcomb, V.B., H. Vogel, and P. Hasty, *Deletion of Ku80 causes early aging independent of chronic inflammation and Rag-1-induced DSBs.* Mech Ageing Dev, 2007. **128**(11-12): p. 601-8.
81. Gullo, C., et al., *The biology of Ku and its potential oncogenic role in cancer.* Biochim Biophys Acta, 2006. **1765**(2): p. 223-34.
82. Wilson, C.R., et al., *Expression of Ku70 correlates with survival in carcinoma of the cervix.* Br J Cancer, 2000. **83**(12): p. 1702-6.
83. Rashmi, R., S. Kumar, and D. Karunagaran, *Ectopic expression of Bcl-XL or Ku70 protects human colon cancer cells (SW480) against curcumin-induced apoptosis while their down-regulation potentiates it.* Carcinogenesis, 2004. **25**(10): p. 1867-77.
84. Omori, S., et al., *Suppression of a DNA double-strand break repair gene, Ku70, increases radio- and chemosensitivity in a human lung carcinoma cell line.* DNA Repair (Amst), 2002. **1**(4): p. 299-310.
85. Castle, V., et al., *Ku70 acetylation in neuroblastoma pathogenesis and therapy.* Trans Am Clin Climatol Assoc, 2010. **121**: p. 183-91; discussion 191.
86. Brodeur, G.M. and R. Bagatell, *Mechanisms of neuroblastoma regression.* Nat Rev Clin Oncol, 2014. **11**(12): p. 704-13.
87. Li, Y., et al., *Bax-inhibiting peptide protects cells from polyglutamine toxicity caused by Ku70 acetylation.* Cell Death Differ, 2007. **14**(12): p. 2058-67.

88. Subramanian, C., et al., *CREB-binding protein regulates Ku70 acetylation in response to ionization radiation in neuroblastoma.* Mol Cancer Res, 2013. **11**(2): p. 173-81.
89. Cohen, H.Y., et al., *Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis.* Mol Cell, 2004. **13**(5): p. 627-38.
90. Chen, C.S., et al., *Histone deacetylase inhibitors sensitize prostate cancer cells to agents that produce DNA double-strand breaks by targeting Ku70 acetylation.* Cancer Res, 2007. **67**(11): p. 5318-27.
91. Abdel-Rahman, W.M., et al., *Role of BAX mutations in mismatch repair-deficient colorectal carcinogenesis.* Oncogene, 1999. **18**(12): p. 2139-42.
92. Spilianakis, C., J. Papamatheakis, and A. Kretsovali, *Acetylation by PCAF enhances CIITA nuclear accumulation and transactivation of major histocompatibility complex class II genes.* Mol Cell Biol, 2000. **20**(22): p. 8489-98.
93. Sundaresan, N.R., et al., *SIRT3 is a stress-responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku70.* Mol Cell Biol, 2008. **28**(20): p. 6384-401.
94. Subramanian, C., et al., *HDAC6 deacetylates Ku70 and regulates Ku70-Bax binding in neuroblastoma.* Neoplasia, 2011. **13**(8): p. 726-34.
95. Hurwitz, J.L., et al., *Vorinostat/SAHA-induced apoptosis in malignant mesothelioma is FLIP/caspase 8-dependent and HR23B-independent.* Eur J Cancer, 2012. **48**(7): p. 1096-107.
96. Lee, Y.S., et al., *The cytoplasmic deacetylase HDAC6 is required for efficient oncogenic tumorigenesis.* Cancer Res, 2008. **68**(18): p. 7561-9.
97. Mazumder, S., et al., *Interaction of a cyclin E fragment with Ku70 regulates Bax-mediated apoptosis.* Mol Cell Biol, 2007. **27**(9): p. 3511-20.
98. Maris, J.M., et al., *Neuroblastoma.* Lancet, 2007. **369**(9579): p. 2106-20.
99. Attiyeh, E.F., et al., *Chromosome 1p and 11q deletions and outcome in neuroblastoma.* N Engl J Med, 2005. **353**(21): p. 2243-53.
100. Chang, H.H. and W.M. Hsu, *Neuroblastoma--a model disease for childhood cancer.* J Formos Med Assoc, 2010. **109**(8): p. 555-7.
101. Acosta, S., et al., *Comprehensive characterization of neuroblastoma cell line subtypes reveals bilineage potential similar to neural crest stem cells.* BMC Dev Biol, 2009. **9**: p. 12.
102. Hada, M. and R.P. Kwok, *Regulation of ku70-bax complex in cells.* J Cell Death, 2014. **7**: p. 11-3.
103. Vogel, S., et al., *Cytosolic Bax: does it require binding proteins to keep its pro-apoptotic activity in check?* J Biol Chem, 2012. **287**(12): p. 9112-27.
104. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays.* J Immunol Methods, 1983. **65**(1-2): p. 55-63.
105. Sawada, M., P. Hayes, and S. Matsuyama, *Cytoprotective membrane-permeable peptides designed from the Bax-binding domain of Ku70.* Nat Cell Biol, 2003. **5**(4): p. 352-7.
106. Clifford-Nunn, B., H.D. Showalter, and P.C. Andrews, *Quaternary diamines as mass spectrometry cleavable crosslinkers for protein interactions.* J Am Soc Mass Spectrom, 2012. **23**(2): p. 201-12.
107. Mimori, T., J.A. Hardin, and J.A. Steitz, *Characterization of the DNA-binding protein antigen Ku recognized by autoantibodies from patients with rheumatic disorders.* The Journal of biological chemistry, 1986. **261**(5): p. 2274-8.
108. Featherstone, C. and S.P. Jackson, *Ku, a DNA repair protein with multiple cellular functions?* Mutation research, 1999. **434**(1): p. 3-15.
109. Sawada, M., et al., *Ku70 suppresses the apoptotic translocation of Bax to mitochondria.* Nature cell biology, 2003. **5**(4): p. 320-9.

110. Sawada, M., P. Hayes, and S. Matsuyama, *Cytoprotective membrane-permeable peptides designed from the Bax-binding domain of Ku70.* Nature cell biology, 2003. **5**(4): p. 352-7.
111. Subramanian, C., et al., *Ku70 acetylation mediates neuroblastoma cell death induced by histone deacetylase inhibitors.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(13): p. 4842-7.
112. Subramanian, C., et al., *CREB-binding protein is a mediator of neuroblastoma cell death induced by the histone deacetylase inhibitor trichostatin A.* Neoplasia, 2007. **9**(6): p. 495-503.
113. Subramanian, C., et al., *Ku70 acetylation mediates neuroblastoma cell death induced by histone deacetylase inhibitors.* Proc Natl Acad Sci U S A, 2005. **102**(13): p. 4842-7.
114. Subramanian, C., et al., *Histone deacetylase inhibition induces apoptosis in neuroblastoma.* Cell Cycle, 2005. **4**(12): p. 1741-3.
115. Vishnudas, V.K. and J.B. Miller, *Ku70 regulates Bax-mediated pathogenesis in laminin-alpha2-deficient human muscle cells and mouse models of congenital muscular dystrophy.* Hum Mol Genet, 2009. **18**(23): p. 4467-77.
116. Choudhary, C., et al., *Lysine acetylation targets protein complexes and co-regulates major cellular functions.* Science, 2009. **325**(5942): p. 834-40.
117. Yuan, J., M. Lipinski, and A. Degterev, *Diversity in the mechanisms of neuronal cell death.* Neuron, 2003. **40**(2): p. 401-13.
118. Kole, A.J., R.P. Annis, and M. Deshmukh, *Mature neurons: equipped for survival.* Cell Death Dis, 2013. **4**: p. e689.
119. Kristiansen, M. and J. Ham, *Programmed cell death during neuronal development: the sympathetic neuron model.* Cell Death Differ, 2014. **21**(7): p. 1025-35.
120. Edsjo, A., et al., *Neuroblastoma cells with overexpressed MYCN retain their capacity to undergo neuronal differentiation.* Lab Invest, 2004. **84**(4): p. 406-17.