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Abstract

Title: Characterization of the roles of the Hax-1 variants in apoptosis

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HS-1-Associated-protein-X-1 (Hax-1) is a family of ubiquitously expressed proteins ranging in size from ~26 to ~35 kDa resulting from alternative splicing of the HAX1 gene (1,2). The prototypical Hax-1 variant 1 is a ~35 kDa protein, expressed in both humans and rodents. Hax-1 contains an NH2-terminal acidic box, followed by two predicted Bcl-2 homology domains, BH1 and BH2, a PEST motif, a predicted COOH-terminal transmembrane (TM) domain, and an Integrin β6 binding domain (3). Studies on Hax-1 have mainly focused on variant (v) 1, demonstrating its anti-apoptotic properties, which have been confirmed by its overexpression in HeLa cells, HEK293 cells, and cardiomyocytes promoting cell survival after exposure to different apoptotic stimuli (4-7). Hax-1 variant 1 has also been shown to be overexpressed in several diseases including psoriasis, melanoma, breast, and lung cancers (8,9).

The presence of at least four Hax-1 variants expressed in rat heart both before and after insult was confirmed, some of which exhibited differential expression before and after induction of myocardial infarction (MI), with v2 being highly up-regulated post-insult. Contrary to anti-apoptotic rat and human v1, overexpression of rat v2 or human v4 (the human homologue of rat v2) in epithelial cells exacerbated cell death by 30% following H2O2 treatment, compared to control vector. Co-expression of rat v1 and v2 or human v1 and v4 neutralized the protective effects of rat and human v1, and the pro-
apoptotic effects of rat v2 and human v4 by modulating cytochrome C release. This is, at least partly, mediated by the ability of Hax-1 proteins to form homotypic and heterotypic dimers with binding affinities in the nM range. The binding region supporting these interactions lies between amino acids 97-278, which are shared by nearly all Hax-1 proteins, indicating that additional factors regulate the preferential formation of Hax-1 homo- or hetero-dimers. Our studies are the first to show that Hax-1 is a family of anti- and pro-apoptotic regulators that may modulate cell survival and death through homo- or hetero-dimerization.
Characterization of the Roles of the Hax-1 Variants in Apoptosis

By
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Chapter 1: Introduction

1.1 Background information

HS-1-Associated-protein-X-1 (Hax-1) is a family of ubiquitously expressed proteins ranging in size from ~26 to ~35 kDa that result from alternative splicing of the single HAX1 gene (1,2). The prototypical Hax-1 variant 1 is a ~35 kDa protein, expressed in both humans and rodents. Early on, it was postulated that Hax-1 contains an NH$_2$-terminal acidic box, consisting of Asp (D) and Glu (E) residues, followed by two purported Bcl-2 homology domains, BH1 and BH2, a PEST motif, a predicted COOH-terminal transmembrane (TM) domain, and an Integrin β6 binding domain(3). Recently though, the existence of the BH1, BH2, and TM domains has been disputed on the basis of data obtained by sequence analysis and structure prediction (10,11).

Hax-1 was suggested to be involved in promoting cell survival, based on its homology and predicted structural similarity to Bcl-2, which is a well characterized anti-apoptotic protein. The anti-apoptotic role of Hax-1 variant 1 has since been confirmed in different experimental and disease models. Consistent with this, ectopic expression of Hax-1 variant 1 in HeLa cells, HEK293 cells and cardiomyocytes promotes cell survival, following exposure to different apoptotic stimuli (4-7). More importantly, overexpression of Hax-1 variant 1 has been found in psoriasis, a severe inflammatory disease characterized by increased proliferation and diminished apoptosis of keratinocytes(9), as well as in melanoma, breast and lung cancers (8).
Hax-1 variant 1 has been reported to interact with an increasingly diverse array of proteins (3-5,7,12-15), indicating that it might exert its anti-apoptotic activities through different pathways. Thus, it has been documented that Hax-1 directly binds initiator caspase-9 inhibiting its activation (6,7), and SarcoEndoplasmic Reticulum Ca$^{2+}$ ATPase (SERCA) pump and its regulator phospholamban (PLN) which modulate Ca$^{2+}$ homeostasis (7,14,16,17). Hax-1 is also involved in the processing and activation of HtrA2 by the mitochondrial protease PARL (18). Active HtrA2 prevents the accumulation of pro-apoptotic Bax in the outer mitochondrial membrane (18), which results in reduced cytochrome C release from the mitochondria and thus decreased apoptosis. This is in no way an exhaustive list of interacting partners of Hax-1, or of its potential mechanisms of action.

While the importance of Hax-1 in regulating cell survival and death has been demonstrated, its exact mechanism of action still remains unclear. This is complicated by the presence of multiple, functionally diverse Hax-1 binding partners, and the existence of many structurally distinct Hax-1 splice variants (10,19).

1.2 Hax-1 in health and disease

Aberrant expression of Hax-1 has been shown to be associated with several diseases. Notably, Hax-1 variant 1 overexpression has been linked to psoriasis and certain types of cancers, both of which are partially characterized by cells resistance to apoptosis (8,9,20,21). Both tumorigenesis and progression of psoriatic hyperplasia are exacerbated by cells reduced susceptibility to apoptosis (22-24). It could therefore be anticipated that the reduced susceptibility to apoptosis seen in both cancers and psoriasis
are due to the increased levels of the anti-apoptotic Hax-1 variant 1 also present in these tissues.

The *HAX1* gene was identified as being highly deregulated following the development of psoriasis by differential display of reverse-transcribed mRNA polymerase chain reaction (DDRT-PCR) (9). This finding was supported by northern blot analysis of Hax-1 levels in normal, lesional, and non-lesional psoriasis samples taken from different patients. It was found that Hax-1 expression was increased by as much as 16 fold in lesional psoriasis, compared to non-lesional psoriasis. Levels of Hax-1 were also reported to be higher in non-lesional psoriasis relative to normal tissue (9). Hax-1 was subsequently found to not only be upregulated at the transcript level, but also at the protein level as well. Hax-1 was upregulated 13 fold in HaCaT cells, a model of psoriatic keratinocytes because of their high proliferative and anti-apoptotic activity, compared to normal human keratinocytes (9). It was also shown that using anti-sense mRNA to inhibit Hax-1 expression in the same HaCaT cells resulted in increased apoptosis and caspase activation (9). These findings suggest that Hax-1 is responsible for the reduction in apoptosis in both lesional and non-lesional psoriatic keratinocytes and is therefore involved in regulating the progression of the disease.

Levels of the Hax-1 transcript were also found to be significantly higher in several types of cancer, including: hepatoma, lymphoma, melanoma, leukemia, myeloma, as well as breast, lung and hematopoietic malignancies (25). Importantly, it was determined that the amount of increase in the Hax-1 transcript correlated to the size and grade of tumor, meaning that higher amounts of Hax-1 were seen as the cancer progressed (8). The increase in Hax-1 transcript expression in certain cancers was supported by an
increase of Hax-1 at the protein level in several malignant cell types, including the highly metastatic MV3 and BLM melanoma cell lines as well as the slowly growing SK-Mel-28 melanoma cell line (9). This pattern was also confirmed in a more recent study, which showed overexpression of Hax-1 protein in human melanoma A375 cells when compared to control non-malignant HaCaT human epidermal keratinocytes (21). A significant upregulation of Hax-1 was also seen in several human premalignant epithelial dysplastic, oral squamous cell carcinoma (SCC), and breast cancer tissue samples by immunohistochemistry (8,26).

Our lab then examined the expression profile of different Hax-1 variants in breast, skin, and colon cancer cell lines via immunoblot. Two breast adenocarcinoma cell lines were used, MCF7 (estrogen-receptor positive) and MDA-MB-231 (estrogen-receptor negative), as well as the skin squamous cell cancer line SCC13, and the colon adenocarcinoma cell line LS174T. In order to more effectively study different Hax-1 variants, three different Hax-1 antibodies were used: one monoclonal (obtained from BD Biosciences) and two polyclonal (either obtained from Santa Cruz, or homemade). The presence of a 35 kDa band, corresponding to Hax-1 variant 1, was seen in all normal and cancer cells studied, while additional immunoreactive bands ranging in size from 25-75 kDa were also detected in some samples. The larger immunoreactive bands likely represent larger uncharacterized Hax-1 variants, post translational modifications, or strong dimers or multimers of the smaller Hax-1 variants that were not disrupted by the denaturing conditions of the gel. Interestingly, protein levels of Hax-1 variant 1 were significantly increased in skin (SSC13) and colon (LS174T), but not in breast (MCF7 and MDA-MB-231), cancer cell lines (19). A similar expression profile was found for most
of the larger immunoreactive bands as well, indicating that they are more prevalent in skin and colon cancer (19). Interestingly, the smaller 25 kDa band was only detected in normal breast cells (MCF10A) and not in any of the cancer cell lines, suggesting that it may function in an opposite manner to the anti-apoptotic Hax-1 variant 1, and may be involved in promoting cell death (19).

There is no direct evidence that Hax-1 expression is responsible for cancer progression, but it is likely that Hax-1 regulates the survival of cancer cells through an apoptotic mechanism. It was shown that blocking Hax-1 expression with small inhibitory RNA (siRNA) results in significantly increased levels of apoptosis in melanoma cells (21). It was also shown that blocking Hax-1 expression with siRNAs inhibits integrin αvβ6-dependent migration of squamous cell carcinoma (SCC) and tumor invasion, demonstrating that Hax-1 may be involved in the migration and invasion of cancer (26). Taken together, these findings suggest that Hax-1 may allow cancer cells to resist apoptosis and might increases αvβ6-mediated migration and invasion of cancer cells. Accordingly, Hax-1 may be a critical player in the development, progression, and invasiveness of cancer, which warrants further investigation. Hax-1 has also been shown to affect cell mobility through Ga13-mediated pathways, although Hax-1’s effect on migration and metastasis of cancer through this pathway is still unknown (27).

Hax-1 was also identified as being significantly upregulated in a renal cancer cell line following hypoxia (20). This renal cancer cell line, 786-0, was altered to express the von Hippel-Lindau tumor suppressor (pHVL), which is involved in the regulation of hypoxia induced signaling pathways (20). A five-fold increase in Hax-1 was seen after induction of hypoxia, both in the presence and absence of pVHL (20). These findings
indicate that Hax-1 enhances the adaptive response of cancer cells to hypoxia, thus promoting cancer survival and growth.

While the overexpression of Hax-1 in both cancer and psoriasis is thought to occur after the development of the disease, reduced expression of Hax-1 has been shown to lead to severe congenital neutropenia (SCN) or Kostmann syndrome, and central nervous system (CNS) or neurodevelopmental delays and abnormalities (28-31). Loss of Hax-1 expression is most often due to inherited mutations in the HAX1 gene, specifically single base changes that result in nonsense mutations and insertions or deletions leading to translational frame-shifts and to premature stop codons (31,32). Currently, 10 different mutations have been identified in HAX1 in humans, most of which are located within exons 2, 3, or 5 of the HAX1 gene. Individuals with mutations in both parental alleles inevitably develop SCN, but do not always show neurological symptoms (28,31,32). Another study focused on two specific HAX1 mutations, R86X and R126fsX128, where ‘X’ indicates a stop codon. Individuals who are homozygous for either of these mutations completely lack Hax-1 protein(s) (29). Patients with heterozygous mutations, affecting only a single parental allele, have no noticeable phenotype, and are able to produce the Hax-1 protein(s) normally (29,33). Mutations in the HAX1 gene are therefore responsible for an autosomal recessive pattern of inheritance for SCN, which has been supposed for over 50 years, but Hax-1 was only very recently identified as a major contributing factor.

The locations of the aforementioned mutations in the HAX1 gene are particularly important. This is due to the existence of multiple Hax-1 isoforms, some of which may have overlapping or antagonistic functions. Certain mutations may inactivate some Hax-
1 variants but not others because of splice variant differences. There are at least seven known Hax-1 splice variants in both human and rat, where most of the splicing events involve the inclusion or skipping of exon 1, intron 1, exon 2, or intron 2 (1). Human Hax-1 variant 4 differs from variant 1 in that it lacks the 5’ portion of exon 2, a region where 5 common Hax-1 mutations have been identified (31,32). Therefore, these mutations only affect Hax-1 variant 1, and not variant 4. Patients with mutations only effecting Hax-1 variant 1 still exhibit SCN, indicating that variant 1 has an essential role on hematopoiesis and apoptosis in neutrophils, and suggests that variant 4 may have another function (28,31). In SCN patients with mutations affecting both Hax-1 variant 1 and 4, neurological symptoms consisting of cognitive impairment, neurodevelopmental delays and neurodegeneration are seen, whereas SCN patients with mutations in the HAX1 gene affecting only variant 1 do not exhibit these neurological symptoms (31,32).

Hax-1 deficiencies also have aberrant effects at the cellular level, including abnormally increased dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$), an increase in apoptosis of hematopoietic cells such as myeloid cells and neutrophils, and an increased release of cytochrome C from the mitochondria (30). Hax-1 deficiency has also been shown to increase apoptosis in fibroblasts, but to a lesser extent than in hematopoietic cells, demonstrating that Hax-1 may be of more importance in some cell types (30,34).

1.3 Hax-1 expression during apoptosis

Since Hax-1 is an apoptosis regulator, its expression is tightly controlled during both cell survival and death. Much like the upregulation of Hax-1 variant 1 leading to increased cellular resistance to apoptosis, as seen in some cancers and psoriasis, Hax-1
levels are also altered during cell death but in an opposite fashion. It has been shown that levels of Hax-1 variant 1 are down regulated during apoptosis. Specifically, a dose dependent decrease in Hax-1 variant 1 was seen after cisplatin, H₂O₂, or etoposide exposure in HEK293 and Jurkat cells (34,35). Downregulation of Hax-1 during apoptosis is partly mediated by HtrA2/Omi, a mitochondrial protease (34). Interestingly, Hax-1 has been proposed to mediate the activation of HtrA2/Omi in the inter mitochondrial membrane space, thus preventing apoptosis through the removal of mitochondrial pore forming protein BAX (18). However, once apoptosis has been initiated, the active HtrA2 cleaves Hax-1 and is released to the cytoplasm, along with other proteins including cytochrome C (34). It was also shown that the use of Ucf-101, an HtrA2 inhibitor, prevents the downregulation of Hax-1 during apoptosis, suggesting that HtrA2 is necessary for this reduction in Hax-1 (34). It has also been shown that caspase 3 plays a role in the reduction of Hax-1 during apoptosis, and it was found that Hax-1 was not downregulated in caspase 3 deficient mice following etoposide treatment (35). These studies show that Hax-1 levels during apoptosis may be regulated by more than one mechanism, however the absence of either HtrA2 or Caspase 3 are sufficient to prevent Hax-1 downregulation during apoptosis.

1.4 Hax-1 expression in humans

The Hax-1 protein is ubiquitously expressed in humans, although at varying levels in different tissues (9,28,36). Based on northern blot data, it was found that Hax-1 expression is highest in the heart and colon, followed by skeletal muscle, lung, liver, and the pancreas (3). Another study found Hax-1 mRNA in all human tissues tested, with the highest levels in skeletal and cardiac muscle (9). Lower levels of Hax-1 mRNA were
found in the brain and pancreas, where the kidney, liver, lung and placenta contained the least amounts (9). Across all studies on Hax-1 expression in human tissues, higher levels of Hax-1 were found in cardiac and skeletal muscle relative to other tissues tested. However, the relative amounts of Hax-1 reported in other tissues varied considerably from one study to another, suggesting that other factors such as age, race, gender, or disease conditions may impact Hax-1 expression in most tissues (3,9,37).

There are currently eight known splice variants of the human HAX1 gene, mostly due to splicing within the first two exons, and several more that have yet to be characterized (1,2,10,38). Human Hax-1 is predicted to contain an NH2-terminal acidic box, consisting of Asp (D) and Glu (E) residues, followed by two purported Bcl-2 homology domains, BH1 and BH2. There are 4 different BH domains, shared by members of the Bcl-2 family of apoptosis regulators. In general, the BH1 and BH2 domains confer an anti-apoptotic function, while the BH3 and BH4 domains tend to confer a pro-apoptotic function. The BH domains are followed by a PEST motif, which acts as a degradation signal, and is found in short lived proteins or those under strict regulatory control. The C-terminus contains a predicted COOH-terminal transmembrane (TM) domain. Despite the existence of many splice variant transcripts, only two protein isoforms have been identified so far. These are predicted to be the ~35 kDa variant 1 (NM_006118) and the ~32 kDa variant 4 (NM_001018837) (7,31). The Hax-1 variant 4 transcript is similar to that of variant 1, except that it lacks the 5’ end of exon 2 (10). Since the reading frame of the transcripts stay intact, this results in a 48 amino acid deletion in variant 4 consisting of a predicted acidic box and a BH1 (BCL2 family homology domain 1) domain (10). These discoveries led to the characterization of the
expression profile and abundance of these two variants in different human tissues. Variant specific qRT-PCR was used to determine the expression levels of the two variants in twenty different tissues (37). While both Hax-1 variant 1 and 4 transcripts were detected in all tissues analyzed, their RNA levels varied greatly between tissues, and the expression profile of each variant was unique (37). Interestingly, the transcript levels of Hax-1 variant 1 were ~20 fold higher than the levels of variant 4 in all tissues analyzed (37). Of important note is the near absence of variant 4 expression in bone marrow and peripheral blood cells, as these are particularly vulnerable to apoptosis during Hax-1 variant 1 deficiency (29-31,39).

1.5 Hax-1 expression in rodents

Like in humans, Hax-1 is differentially expressed in various tissues in both rats and mice. In rats, the testis have by far the highest levels of Hax-1 transcript, followed by liver, cardiac muscle, and skeletal muscle; with lower expression in other tissues (1). Similarly, mice have high Hax-1 transcript expression in the testis but also have high expression in the kidney; with liver, skeletal muscle, and cardiac muscle exhibiting slightly less Hax-1, and other tissues the least (1,38). Interestingly, both of these studies found that Hax-1 protein levels did not correlate to transcript levels. In the rat, protein levels of Hax-1 are relatively the same among testis, liver, and brain, despite having significantly different transcript levels between the tissues (1). In mice, discrepancies between transcript and protein levels of Hax-1 were particularly apparent in the brain, heart, and testis (38). Amounts of Hax-1 protein in the brain were much higher than anticipated, based on transcript levels, while protein levels in the heart and testis were lower than what would be predicted (38). These results indicate that there is significant
posttranscriptional or posttranslational regulation of Hax-1 in both rats and mice. Since Hax-1 contains a PEST motif, it is also possible that there are tissue specific differences in degradation rates of Hax-1. At this time, similar studies have not been carried out in humans, so the tissue specific transcript to protein correlation for Hax-1 is still unknown.

There are currently seven characterized Hax-1 splice variants in the rat (1). Like in humans, these variants are generated from the splicing of a single HAX1 gene with the major splicing events occurring in intron 1, exon 1, and exon 2 (1). Specifically, the retention of intron 1, exclusion of exon 1, or skipping of parts of exon 2 result in the seven known splice variants (1). While none of these splicing events result in a frame shift, translation can begin either in intron 1, exon 1, or exon 2 (1). Much like the human variant 1, rat Hax-1 variant 1 is predicted to contain an N-terminus mitochondrial targeting sequence (or nuclear localization sequence in other variants), acidic box, consisting of Asp (D) and Glu (E) residues, followed by two purported Bcl-2 homology domains, BH1 and BH2, a PEST motif, a predicted COOH-terminal transmembrane (TM) domain, and an Integrin β6 binding domain (3) (Fig. 1.1). The expression of the ~35 kDa Hax-1 variant 1 and ~32 kDa variant 2 (rat variant 2 is homologous to human variant 4) have been confirmed at the protein level in several rat tissues (1,40). Similar to the human Hax-1 variants 1 and 4, rat Hax-1 variant 2 differs from variant 1 in that it lacks part of exon 2, resulting in the loss of the predicted BH1 and BH2 domains (1). Three additional, yet to be identified, Hax-1 variants have also been found at the protein level in several rat tissues (40).
Figure 1.1: Alignment of rat and human Hax-1 variant 1 amino acid sequences.
Both rat and human Hax-1 variant 1 are predicted to be made up of an N-terminus mitochondrial targeting sequence (MTS), acidic box, two predicted Bcl-2 homology domains, BH1 and BH2, a PEST motif, a predicted COOH-terminal transmembrane (TM) domain, and an Integrin β6 binding domain(3). Also shown in this figure are the predicted Caspase 9 and Phospholamban (PLN) binding sites, which are also shared by both the rat and human Hax-1 variant 1.
Mice, unlike rats and humans, only have two known Hax-1 splice variants referred to as Hax-1 and Hax-1xs (2,41). Interestingly, Hax-1 and Hax-1xs are differentially regulated in various tissues (2). Under normal conditions mouse Hax-1 is ubiquitously expressed, but the smaller variant, Hax-1xs, is only expressed at low levels in the kidney, thymus, and lung (2). It was found that Hax-1xs, but not Hax-1, is significantly upregulated at both the transcript and protein levels following burn injury (41). The specific upregulation of Hax-1xs following burn injury suggests that it may be involved in stress condition signaling, like apoptosis. This finding also suggests the possibility of differential regulation of the rat and human Hax-1 variants during disease or stress, and also suggests that some Hax-1 splice variants may function differently to the prototypical variant 1.

1.6 Subcellular localization of Hax-1

In order to help elucidate the subcellular mechanism by which Hax-1 affects apoptosis, several studies were conducted to characterize its subcellular distribution (3-7,42). It is important to note that most of these studies looked at localization of exogenous Hax-1 variant 1 in various cell lines. Currently, a major limitation in studying Hax-1 is the lack of variant specific antibodies, making the study of specific endogenous Hax-1 variants very difficult. Hax-1 variant 1 was first shown to localize to the mitochondria in COS7 cells, and co-localized with Bcl-Xl which is an antiapoptotic mitochondrial membrane protein (3). Other studies found Hax-1 variant 1 in the mitochondrial fraction of HeLa cells and found that Hax-1 and Mitotracker were similarly distributed in HeLa and HEK293 cells (4,5).
While exogenous Hax-1 variant 1 is predominantly localized at the mitochondria, it has been shown that lesser amounts are also found at the endoplasmic reticulum (ER) and nuclear membrane (3). Other studies showed exogenous Hax-1 variant 1 localizing at the ER with K15 protein as well as at the mitochondria (4). Surprisingly, more recent studies have shown exclusive cytoplasmic localization of exogenous Hax-1 variant 1 in COS7 cells and strong ER localization in HeLa cells, which contradicts the original study by Suzuki et al (3,36). Interestingly, exogenous rat Hax-1 variant 1 (which is homologous to human variant 1) was found in both the mitochondria and cytoplasm of transfected adult rat cardiac myocytes, adding even more complexity to subcellular localization of Hax-1 (6). While some technical differences exist between these studies, like the use of GST tagged Hax-1, the Hax-1 antibodies used, or the co-expression of Hax-1 and another construct, they illustrate the complexity of Hax-1 and suggest that Hax-1 has tissue or cell type specific distribution.

Some work has been done on the localization of endogenous Hax-1 in human cell lines and tissue. Findings are fairly inconsistent, but endogenous Hax-1 variant 1 has been found in the mitochondria, nucleus, and endoplasmic reticulum (ER) / sarcoplasmic reticulum (SR) of several cell and tissue types (13,40). One study found exclusive mitochondrial localization for endogenous Hax-1 variant 1, while another study found Hax-1 variant 1 in the cytoplasm of HEK293 cells, in the nuclear fraction of DG75 B-lymphoma cells, and exclusive mitochondrial distribution in an N2a neuroblastoma cell line (34,38,42). Cell fractionation of whole tissues also showed the presence of endogenous Hax-1 at the mitochondria, nucleus, and ER/SR (13,40,43). Another study found Hax-1 in both the mitochondrial and nuclear fractions of rat testis, but found the
cytoplasm to be lacking Hax-1 (43). Yet another study showed the presence of Hax-1 in both the mitochondrial and ER fractions of rat liver, but not in the cytoplasm or the nuclear fractions (13). Our lab previously showed that Hax-1 was present at both the mitochondria and SR of rat striated muscle, and was most highly concentrated where mitochondria and the SR were in close proximity (40). The distribution patterns of Hax-1 are too widely varying to draw conclusions about the localization of endogenous Hax-1 compared to exogenous Hax-1. Again, these results suggest that the localization of Hax-1 is cell type and tissue specific, indicating that it may play several different roles.

An additional complicating factor in studying the subcellular localization of Hax-1 is the existence of multiple variants. Both Hax-1 variant 1 and variant 2, ~35 kDa and ~32 kDa respectively, were identified in the testis, brain, liver, and striated muscle of rats using one dimensional gel electrophoresis (1,40). Three additional, less abundant, protein isoforms have also been found in the rat; ~50 kDa and ~33 kDa isoforms in striated muscle and a ~27 kDa isoform in the liver, brain, and testis (1,40). The ~33 kDa isoform is predicted to represent Hax-1 variant 5 and the ~27 kDa isoform is predicted to represent variant 6 (40). These predictions have not been confirmed experimentally, but are based on the sizes of the known Hax-1 variants. Interestingly, the ~50 kDa protein does not correspond in size to any of the known rat Hax-1 variants and may represent a posttranslationally modified isoform, a novel uncharacterized isoform, antibody cross reactivity, or a strong Hax-1 dimer that was not disrupted by the denaturing conditions of the gel. Both of the predominant rat Hax-1 variants, the ~35 kDa and ~32 kDa variants 1 and 2, were found in the SR and mitochondrial membranes of striated muscle (40). Interestingly, the ~33 kDa isoform was seen only at the mitochondria, while the ~50 kDa
isoform was found only at the SR of the same striated muscle (40). The variation in reports of Hax-1 subcellular localization may be due to the presence of multiple Hax-1 isoforms with different localizations. This further supports the hypothesis that several Hax-1 variants may serve functions within the cell different from the prototypical variant 1.

To further compound the complexity of the subcellular localization of Hax-1, it has been shown that a redistribution of Hax-1 occurs in breast cancer (8). In normal, non-tumorigenic, breast epithelium Hax-1 is only found within the cytoplasm and not at any other organelles (8). Interestingly, Hax-1 is found in the cytoplasm, ER, and the nucleus of breast cancer epithelium (8). The presence of Hax-1 at the ER and the nucleus seems correlated, as Hax-1 was never seen at just the ER or nucleus alone (8). This study suggests that Hax-1 is involved in transcriptional regulation of breast cancer cells, which is also supported by a change in Hax-1 expression patterns as previously discussed (38, 43, 44).

Hax-1 has been shown to have many different binding partners, and some of these proteins have been shown to alter the subcellular localization or relative abundance of Hax-1 in different cellular compartments. For example, Hax-1 was shown to redistribute to the plasma membrane in the presence of the bile salt export protein (BESP) (13). When both Hax-1 variant 1 and BESP are overexpressed in Madin-Darby Canine Kidney Type II epithelial cells (MDCK-II), Hax-1 translocated from the cytoplasm and accumulated at the apical cell membrane and co-localized with BESP (13). It was also shown that when polycystic kidney disease protein 2 (PKD2) and human Hax-1 variant 1 were both overexpressed in HeLa cells, Hax-1 was again translocated from the cytoplasm
to the cell membrane (12). PKD2 is a calcium binding protein involved in the formation of cell to cell and cell to substrate contacts, and is normally found at the cell periphery (12). It is likely that the interaction between Hax-1 variant 1 and PKD2 is responsible for the translocation of Hax-1 to the cell membrane. Additionally, co-expression of human Hax-1 variant 1 and cortactin resulted in the translocation of Hax-1 from the cytoplasm to the cell periphery, specifically to cellular projections, in COS7 cells (12). Cortactin is an actin binding protein important for cytoskeletal movement, such as the cell migration, lamellapodia formation, endocytosis, synaptogenesis, and invasiveness. It was also shown that the translocation of Hax-1 to the plasma membrane was significantly enhanced in actively growing and moving cells (12). These studies were the first to show the dynamics of Hax-1 localization in non-disease state and suggest that Hax-1 plays a role in the reorganizing of the cytoskeleton and possibly in motility and migration.

Localization of Hax-1 to multiple cell compartments has inspired the investigation into targeting motifs within the Hax-1 protein. It was first suggested that amino acids 1-26 of Hax-1 variant 1 make up a mitochondrial targeting sequence (1). This sequence was identified by PSORT prediction software, and is present in the human, mouse, and rat isoforms in variant1, rat Hax-1 variant 2, and human Hax-1 variant 4 (the human variant 4 is the homologue of rat Hax-1 variant 2) (1). Accordingly, this N-terminal domain is likely to be responsible for targeting Hax-1 to the mitochondria in all three species. This was confirmed using a series of N-terminal Hax-1 deletion constructs, showing that the first 59 amino acids are required to maintain Hax-1’s mitochondrial localization (40). It was also found that the phospholamban (PLN) binding site in the C-terminus of Hax-1 is required for Hax-1’s ER/SR localization (40). This is consistent
with an earlier finding showing that Hax-1 translocates to the ER when co-expressed with PLN and is also supported by the finding that Hax-1 is lost from the ER in the absence of PLN (7,40).

In order to correlate the presence of the mitochondrial targeting sequence with subcellular localization, the distribution of several rat Hax-1 variants was studied. At least seven rat Hax-1 splice variants have been identified, but only variants 1 and 2 contain the predicted mitochondrial targeting sequence, which is encoded by exon 1 (1). It was found that Hax-1 variants 1 and 2, which contain the mitochondrial targeting sequence, predominantly localize at the mitochondria, with minimal ER/SR localization (40). As predicted, Hax-1 variants 3, 4, and 5, which lack the mitochondrial targeting sequence, were present at the highest levels at the ER/SR, although they were still found at the mitochondria in much lower levels (40). While rat Hax-1 variants 6 and 7 were not included in this study, they both lack the predicted mitochondrial targeting sequence and would likely localize to the ER/SR. Extrapolating this data to other species, we would expect that human Hax-1 variants 1, 3, 4, 5, 6 and mouse Hax-1 and Hax-1xs, would predominantly localize at the mitochondria, since these proteins all contain the mitochondrial targeting sequence. Human Hax-1 variants 2, 7, and 8 would preferentially localize to the ER/SR, as they either do not contain or do not express the mitochondrial targeting sequence portion of exon 1 (1).

It has also been predicted that the N-terminal region of rat Hax-1 variants 3 and 4 contain a nuclear localization signal, however its functionality has not been experimentally confirmed (1). This sequence is encoded by intron 1a, which is present in rat variants 3 through 7, but only predicted to be translated in variants 3 and 4 (1). Hax-1
has been found in the nucleus in human, rat, and mouse cells and tissues, and has been shown to directly interact with the 3’ untranslated region (UTR) of human vimentin mRNA and with rat polymerase β mRNA (43,44). Although it has not been confirmed, these results suggest that a nuclear targeting signal present in Hax-1 is responsible for its nuclear localization.

While the anti-apoptotic function of Hax-1 variant 1 has been well established, the function of the other Hax-1 splice variants is still unknown. The other splice variants might also be anti-apoptotic, like variant 1, but have different subcellular localizations, or could have different functions entirely. We sought to characterize what, if any, role some of the other rat Hax-1 variants play in apoptosis and how the expression profile of the Hax-1 variants determines cell fate.

The mechanism by which Hax-1 variant 1 confers its anti-apoptotic function is still unknown. While a diverse array of Hax-1 binding partners have been identified, any proposed mechanisms remain untested. Hax-1 has been shown to interact with proteins involved in calcium regulation, immune response, caspase activation, and the intrinsic apoptosis pathway. More work is needed to determine which of these interactions are necessary for Hax-1 variant 1 to confer its anti-apoptotic function and if any of these interactions could lead to additional functions of the other Hax-1 variants. Based on its interaction with Parl and HtrA2, we hypothesize that Hax-1 is involved in the intrinsic apoptosis pathway and seek to determine how Hax-1 variant 1 prevents apoptosis.
Chapter 2: Competition through dimerization between anti-apoptotic and pro-apoptotic Hax-1 proteins

2.1 Introduction

Studies on Hax-1 have mainly focused on variant (v) 1, demonstrating its anti-apoptotic properties. However, HAX1 is heavily spliced, generating structurally distinct isoforms. We sought to characterize the Hax-1 isoforms expressed in rat heart before and after insult. We confirmed the presence of at least four Hax-1 transcripts in healthy rat cardiac muscle. These exhibited differential expression before and after induction of myocardial infarction, with v2 being up-regulated 12-fold at the transcript level, and 1.5-fold at the protein level, post-insult. Contrary to anti-apoptotic rat and human v1, overexpression of rat v2 or human v4 (the human homologue of rat v2) in epithelial cells exacerbated cell death by 30% following H$_2$O$_2$ treatment, compared to control vector. Co-expression of rat v2 or human v4 with v1 neutralized the protective effects of rat and human v1, and the pro-apoptotic effects of rat v2 and human v4 by modulating cytochrome C release. This is, at least partly, mediated by the ability of Hax-1 proteins to form homotypic and heterotypic dimers with binding affinities ranging from ~3.8 nM for v1 dimers to ~97 nM for v1/v2 dimers. The minimal binding region supporting these interactions lies between amino acids 97-278, which are shared by nearly all Hax-1 proteins, indicating that additional factors regulate the preferential formation of Hax-1 homo- or hetero-dimers. Our studies are the first to show that Hax-1 is a family of anti- and pro-apoptotic regulators that may modulate cell survival and death through homo- or hetero-dimerization.
2.2 Experimental Procedures

Myocardial Infarction (MI)

Frozen lyophilized heart tissue from adult Sprague Dawley rats was generously donated by Dr. William Stanley (University of Maryland, School of Medicine). Heart failure was induced by constriction of the left coronary artery to simulate myocardial infarction (MI), as previously described (45,46). In brief, rats were anesthetized, intubated, and ventilated, and then left coronary artery ligation was performed with a stainless steel clamp, to restrict blood flow to the heart. The clamp was left in place and the chest cavity was closed. Control sham animals underwent the same surgery without artery ligation. Heart tissue was collected from both groups 12 weeks after surgery.

Quantitative RT-PCR

Poly-A mRNA was isolated from rat cardiac tissue subjected to MI or sham surgery using the MicroPoly(A) Purist Kit (Ambion, Life technologies, Grand Island, NY, USA). qRT-PCR reactions were set up in 50μl volume using Bio-Rad iScript and IQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Hax-1 variant specific primers were designed to span exon/intron junctions, with an optimal T_m of 60°C, and a length between 18 and 25 bases. Of the 7 known rat Hax-1 variants, we were able to specifically amplify variants 1, 2, 4 and 6. The primers used were designed according to the published rat variant sequences (accession numbers: NM_181627.2, AY919342.1, and AY291064.2) and were as follows: for v1 Forward primer: 5’-GACCTCGGAGCCACAGAGATC-3’ and Reverse primer: 5’-CCTGGAAGTTCAGGAGAGTGGG-3’, for v2 Forward primer: 5’-
GACCTCGGAGCCACAGAGATC-3’ and Reverse primer: 5’-
CCTGGAAGTTCACTGTCTTCC-3’, for v4 Forward primer: 5’-
GCAAGTCATTGCCACAGAGATC-3’ and Reverse primer: 5’-
CCTGGAAGTTCACTGTCTTCC-3’, and for v6 Forward primer: 5’-
CCTCCATTCTCAGCCACAGAGAT-3’, and Reverse primer: 5’-
CCTGGAAGTTCACTGTCTTCC-3’.

Western blotting

Immunoblots were performed as previously described (19). In brief, samples were run on a 4-112% Bis-Tris gel and transferred to a nitrocellulose membrane. The membranes were washed, blocked, and probed with separate primary and secondary antibodies and developed using an alkaline phosphatase based chemiluminescent system. The only modification to this protocol was that 25 mg of heart tissue were homogenized in NP40 buffer containing 10 mM NaPO₄, 2 mM EDTA, 10 mM NaN₃, 0.9% NaCl, 2% NP40 in the presence of a protease inhibitor cocktail (Roche, Indianapolis IN, USA, product number 11697498001) using a tissue grinder (VWR, Radnor PA, USA, model 47747-366). Protein lysates from three independent experiments were prepared and analyzed. The following primary antibodies were used: Hax-1 (1:1000 dilution, BD Biosciences, Franklin Lakes NJ, USA), His (1:1000 dilution, Santa Cruz Biotechnology, Dallas TX, USA), alpha tubulin (1:1000 dilution, Sigma Aldrich, St. Louis MO, USA), cytchrome C (1:200 dilution, Abcam, Cambridge MA, USA), and GAPDH (1:1000 dilution, Santa Cruz Biotechnology, Dallas TX, USA) followed by goat anti-mouse or donkey anti-rabbit secondary antibody (1:2500 dilution, Jackson Immunoresearch, West Grove PA, USA). Blots were developed using an alkaline phosphatase based
chemiluminescent system (Applied Biosystems, Foster City CA, USA). Relative levels of immunoreactive bands were quantified using Image J software and densitometric analysis (NIH, Bethesda MD, USA).

**Generation of overexpression plasmids**

Overexpression constructs were generated by cloning the complete coding sequence of rat Hax-1 v1 and v2 and human Hax-1 v1 and v4 (Accession numbers: NM_181627.2, AY919342.1, NM_006118.3, and EU190982.1 respectively) into the pIRES2-ZsGreen1 or pIRES2-ZsRed1 Vector (Clontech, Mountain View CA, USA) using the Sac1 and Xma1 restriction sites. The primers used were as follows: for rat v1 and v2 Forward primer: 5’-ACTGGAGCTCATGAGCGTCTTTGAT-3’ and Reverse primer: 5’- ACTGCCCGGGCTATCGGGACCGAAACC -3’. For human v1 the above rat forward primer was used with Reverse primer: 5’- ACTGCCCGGGTACCGGGACCGGAAAC -3’. The authenticity of the generated constructs was confirmed by sequencing (Genewiz Inc, South Plainfield NJ, USA).

**Cell culture and transfections**

HEK293 cells (ATCC, Manassas VA, USA) were cultured in growth media (DMEM supplemented with 10% FBS and 1% Penn/Strep), and maintained in a water-jacketed 5% CO₂ incubator at 37°C. Transfections were performed using Lipofectamine 2000 (Invitrogen, Life technologies, Grand Island, NY, USA) per the manufacturer’s protocol, followed by media change 5 hrs after transfection.
Measurement of cell viability using XTT assay

3x10^5 HEK293 cells were seeded in standard 6-well plates 24 hrs prior to transfection. 24 hrs after transfection, plasmid expression was confirmed by visualization of GFP or RFP fluorescence. Cells were treated with either 2 µM thapsigargin for 24 hrs, 4 µM calcimycin for 24 hrs, or 0.33 mM H_2O_2 for 4 hrs. All treatments were performed in sodium pyruvate-free DMEM in the absence of serum or antibiotics. Cell viability was measured using XTT sodium salt (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt) (Sigma Aldrich, St. Louis MO, USA) according to the manufacturer’s instructions. In this assay, the pale yellow XTT solution is reduced to a bright orange formazan dye by active dehydrogenases in metabolically active cells. The amount of color change is related to cell viability and metabolism using a standard curve.

Evaluation of apoptosis via FACS analysis

Cells were plated, transfected, and treated with the indicated chemicals as for the XTT assay. Following treatment, cells were collected using 0.025% trypsin (Gibco, Life technologies, Grand Island, NY, USA) and prepared for Fluorescence Activated Cell Sorting (FACS) analysis using the Annexin V PE Apoptosis Detection Kit (BD Pharmingen, San Jose California, USA), according to the manufacturers protocol. Cells were labeled with both Annexin V and 7AAd. Annexin V binds to phosphatidylserine which has been exposed on plasma membrane of cells undergoing apoptosis and 7AAD enters damaged cells through holes in the plasma membrane and binds to DNA. Cells that are both Annexin V and 7AAD negative are considered healthy and alive, while cells
that are only Annexin V positive are in early stages of apoptosis, and cells that are positive for both Annexin V and 7AAD are in late stage apoptosis. FACS analysis was performed on a BD FACS Scan Analyzer, by the University of Maryland Flow Core staff. Only GFP positive, RFP positive or GFP/RFP positive cells were included in our analysis, to ensure that only transfected cells were analyzed.

Production of recombinant Hax-1 proteins

6x-His or GST tagged recombinant proteins were generated for use in binding assays. Full length rat Hax-1 v1 and v2 were cloned into the pet30a (Novagen, Darmstadt, Germany) and pGEX (GE Life Sciences, Pittsburgh PA, USA) vectors, for expression of 6x-His- and GST-tagged fusion proteins, respectively. Proteins were expressed in BL21 DE3 cells, following standard techniques. For protein extraction, bacterial pellets from a 100 ml culture were re-suspended in 20 ml STE buffer (10 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, pH 7.5). After one freeze-thaw cycle, lysozyme was added to the cell pellet to a final concentration of 100 µg/ml followed by incubation on ice for 15 minutes. Subsequently, 5 mM DTT and 1% N-lauroylsarcosine were added to the cell slurry followed by sonication for four 10-second bursts on medium power, and centrifugation at 8,900 x g at 4°C for 30 min. The supernatant containing the recombinant protein of interest was removed and 2% Triton X-100/20 mM CHAPS were added, followed by incubation with gentle rocking for 4 hr at 4°C. All samples were dialyzed in cold PBS in the presence of 10 mM NaN₃ and 1 mM EDTA. 6x-His tagged proteins were purified using Ni-NTA beads (Qiagen, Hilden, Germany) and gravity flow columns, following the manufacturer’s protocols. GST-tagged proteins were incubated
with glutathione-Sepharose beads (Novagen, Darmstadt, Germany) as per the manufacturer’s instructions.

_Protein binding via GST-pull down_

GST-tagged rat Hax-1 v1 was bound to glutathione-Sepharose beads, and incubated with 3 µg of His-tagged Hax-1 v1 or v2 overnight at 4°C in pull down buffer (50 mM Tris pH 7.5, 120 mM NaCl, 10 mM NaN₃, 2 mM DTT, and 0.5% Tween). Beads were washed five times with wash buffer (PBS with 10 mM NaN₃, and 0.1% Tween), and bound proteins were eluted with 2x LDS buffer (Invitrogen, Life technologies, Grand Island, NY, USA), followed by boiling at 95°C for 10 min and separation on a 4-12% Bis-tris gel. Our standard western blotting procedure was followed, using a 6x-His tag antibody (SC-803, Santa Cruz Biotechnology Inc, Dallas Texas, USA).

_Protein binding via Surface Plasmon Resonance (SPR)_

Surface plasmon resonance was performed using a BiaCore 3000 instrument, as previously described (47,48). GST tagged recombinant Hax-1 proteins, referred to as ‘ligand’, were attached to a CM5 chip by antibody capture and washed with buffer. His tagged Hax-1 proteins, referred to as the ‘analyte’, were flown over the chip. Binding of the Hax-1 proteins was measured in real time by changes in the angle of reflected light within the chip as the analyte binds the ligand. Analyte concentration ranged from 25-500 nM. Experiments were performed at least three independent times yielding similar results.
Yeast two-hybrid

The Matchmaker Gold yeast two-hybrid system was used (Clontech, Mountain View CA, USA). In brief, full length rat Hax-1 v1 was cloned into the pGBKT7 vector and served as bait. The rat Hax-1 v1 transcript was split into 8 different regions, based on predicted domain structure, which were cloned into the pGADT7 vector and used as preys. The Gold competent cells, provided by the manufacturer which are unable to produce the amino acids Ade, His, Leu, and Trp, were simultaneously transformed with the indicated bait and prey constructs, and plated on the appropriate selective media. Both the prey and bait proteins are expressed in the yeast cell. The bait protein is fused to the Gal4 DNA binding domain and the prey protein is fused to the Gal4 transcription factor activation domain. If the bait and prey proteins bind, it brings the Gal4 binding and activation domains together, and initiates transcription of the AUR1-C, ADE2, HIS3, and MEL1 genes, allowing the yeast to survive on media deficient in select amino acids. Positive interactions were then streaked on high stringency plates for confirmation and evaluation of their relative strength, as described by the manufacturer.

Measurement of intracellular reactive oxygen species (ROS)

The OxiSelect intracellular ROS assay kit (Cell Biolabs, San Diego CA, USA) was used per the manufacturer’s protocol. Cells overexpressing select Hax-1 constructs were treated with 0.33 mM H₂O₂, and intracellular ROS levels were measured after 1 and 4 hrs of treatment. The cells are treated with the membrane permeable dye DCFH-DA. Once inside the cell, esterases convert the dye to DCFH and after treatment with ROS, in
this case H$_2$O$_2$, the ROS interacts with DCFH to convert it to DCF, which is fluorescent. Fluorescence of treated cells is proportional to the amount in intracellular ROS.

*Measurement of Cytochrome C release*

The Cytochrome C Releasing Apoptosis Assay Kit (Abcam ab65311, Cambridge MA, USA) was used per the manufacturer’s instructions. HEK293 cells were transfected with select Hax-1 constructs as described above, and treated with 0.33 mM H$_2$O$_2$. A pilot time course was performed to determine the time point of maximum cytochrome C release after treatment, which was shown to be 2 hours. In this assay, the cell membrane was disrupted and cytosolic and mitochondrial fractions were collected via centrifugation. The cytoplasmic fractions were then analyzed via western blot for cytochrome C content, indicating that cytochrome C was released from the mitochondria during the process of apoptosis.

2.3 Results: Expression levels, apoptotic role, and dimerization of select Hax-1 variants

*Expression profile of select Hax-1 transcripts in the rat heart prior to and post induction of myocardial infarction*

Hax-1 v1 is ubiquitously expressed, albeit at different amounts among different tissues (9). Consistent with this, high transcript levels of Hax-1 v1 have been reported in the mammalian heart (10), whereas no such information is available for other Hax-1 variants. Using quantitative (q) RT-PCR and variant-specific primers, we compared the expression levels of Hax-1 v1, v2, v4, and v6 transcripts in healthy and stressed rat heart (Fig. 2.1A; no variant-specific primers can be designed for v3, v5 and v7). All four
HAX1 transcript variants were readily expressed in healthy rat myocardium, (Fig. 2.1B, black bars). Interestingly, a significant increase of ~12 fold was detected in the transcript levels of v2 twelve weeks after transaortic ligation and induction of myocardial infarction (Fig. 2.1B, grey bars). No statistically significant (p<0.05 was used as the significance threshold) changes in the transcript levels of v1, v4 and v6 were observed in the stressed rat heart (Fig. 2.1B). The up-regulation of v2 transcripts was corroborated by a ~1.5 fold increase at the protein level (Fig. 2.1 C and D) in the treated hearts, compared to sham-operated control hearts, while the protein levels of Hax-1 v1 remained unchanged (Fig. 2.1 C and D).
Figure 2.1: **Expression profile of Hax-1 variants in the rat myocardium prior and post induction of myocardial infarction.** (A) Schematic representation of Hax-1 variants showing predicted protein domains, corresponding exons (shown as light grey boxes), and introns (shown as dark grey boxes). Domain abbreviations are as follows: mitochondrial targeting sequence (MTS), acidic box (EE), Bcl-2 homology domains 1 and 2 (BH1 and BH2), Proline (P), Glutamic Acid (E), Serine (S), Threonine (T) rich motif, (PEST), predicted transmembrane (TM) domain, and Integrin β6 binding domain (β6); numbers on top indicate amino acid residues defining protein domains. The location of the primers that were used for amplification of Hax-1 variants in qRT-PCR is denoted with arrows. Of note, for consistency purposes we adopted the Hax-1 structural organization presented by Suzuki and colleagues (3), although the presence of the BH1, BH2 and TM domains has been recently disputed (11). (B) qRT-PCR analysis of the expression levels of Hax-1 v1, v2, v4, and v6 in rat myocardium prior and 12 weeks post induction of myocardial infarction. Notably, v2 transcript was up-regulated ~12 fold following myocardial infarction (n=3, t-test, p<0.05, error bars show standard error of the mean, SEM). (C) Immunoblot analysis of Hax-1 v1 and v2 in lysates prepared from rat myocardium prior and 12 weeks post induction of myocardial infarction using antibodies to Hax-1. Equal loading was ensured by probing for tubulin. (D) Quantification of the relative protein levels of Hax-1 v1 and v2 with densitometry and Image J software demonstrated that v2 levels are increased by ~1.5 fold 12 weeks post induction of myocardial infarction (n=3, t-test, p<0.03, error bars show SEM).
Figure 2.1: Expression profile of Hax-1 variants in the rat myocardium prior and post induction of myocardial infarction.
Hax-1 v1 and v2 have antagonistic roles in regulating H$_2$O$_2$-induced apoptosis

Given the increased expression levels of Hax-1 v2 in the rat heart following myocardial infarction, we set forth to examine if and how v2 regulates cell death. We used the HEK293 epithelial cells, which have been extensively and reliably used for such studies (49). We transiently transfected HEK293 cells with rat Hax-1 v1, v2, v1 and v2, or control empty vector(s), and subsequently subjected them to different apoptotic stimuli; notably, we routinely observed >80% efficiency for single transfections, and >70% efficiency for co-transfections (Fig. 2.2). We used a variety of apoptotic stimuli, including thapsigargin, which raises cytosolic Ca$^{2+}$ concentration by blocking the activity of SERCA (50), calcimycin, a divalent cation ionophore that allows Ca$^{2+}$ to cross membranes (51), serum starvation (52), and H$_2$O$_2$, which induces oxidative stress and causes cytochrome C release from the mitochondria leading to caspase activation (53). We first measured metabolic activity using the XTT assay, which is indicative of cell viability. Overexpression of Hax-1 v1 or v2 had no effect on cell viability, compared to control cells expressing empty vector, following treatment with thapsigargin (Fig. 2.3A) or calcimycin (Fig. 2.3B), and after serum starvation (Fig. 2.3C). These findings are surprising given the direct association of Hax-1 v1 with major Ca$^{2+}$ regulatory proteins (14,16,54). However, overexpression of Hax-1 v1 protected HEK293 cells from H$_2$O$_2$-induced death by ~1.5 fold (Fig. 2.3D), whereas overexpression of Hax-1 v2 exacerbated cell death by ~0.75 fold (Fig. 2.3D and E) compared to control cells. Importantly, co-transfection of Hax-1 v1 and v2 in HEK293 cells that yielded equivalent amounts of exogenous v1 and v2 proteins (Fig. 2.2), abrogated the anti-apoptotic and pro-death effects of v1 and v2, respectively, following exposure to H$_2$O$_2$ (Fig. 2.3E).
Figure 2.2: Examination of the expression levels of exogenous Hax-1 proteins in HEK293 cells. (A) Immunoblots of lysates collected from HEK293 cells 24 hours post-transfection overexpressing rat Hax-1 v1 (RV1), v2 (RV2), RV1 and RV2, human v1 (HV1), v4 (HV4), or HV1 and HV4, and probed with Hax-1 antibody. The 24-hour time point was used in all of our experiments described in figures 2.2-2.8. Immunoreactive bands are denoted with colored dots for ease of identification: red dot indicates endogenous human v1, green dot points to exogenous rat v1, orange dot denotes exogenous rat v2, blue dot represents endogenous and exogenous human v1, which co-migrate, and yellow dot specifies exogenous human v4. It is of interest to note that HEK293 cells do not express detectable levels of endogenous Hax-1 v4 under normal or stress conditions, and that endogenous and exogenous HV1 migrate slightly faster than exogenous RV1 in our gel system. To ensure equal loading, replica immunoblots were probed with an antibody to tubulin. (B) Fluorescence images of transfected HEK293 cells show ~80% transfection efficiency for single transfections and ~70% efficiency for co-transfections. Single transfected cells express GFP (RV1 and HV1 panels) or RFP (RV2 and HV4 panels), while double transfected cells express both GFP and RFP (RV1+RV2 and HV1+HV4 panels). Images were taken immediately before lysates were collected for (A). Images are representative of all transfection efficiencies.
Figure 2.2: Examination of the expression levels of exogenous Hax-1 proteins in HEK293 cells.
To further confirm these findings, we performed similar experiments using the human homologues of rat v1 and v2, i.e. v1 and v4, respectively. Notably, rat v1 and human v1 share ~84% sequence identity, while rat v2 and human v4 share ~75% identity (10). Similar to the rat variants, overexpression of human v1 in HEK293 cells increased viability by ~1.7 fold following H$_2$O$_2$ treatment (Fig. 2.3E), whereas overexpression of human v4 promoted cell death by ~0.7 fold (Fig. 2.3E). Moreover, co-expression of human v1 and v4 counteracted their individual effects on cell survival (Fig. 2.3E).
Figure 2.3: Effects of Hax-1 variants on cell viability following exposure to different insults. (A–E) HEK293 cells overexpressing rat Hax-1 v1 (RV1) or v2 (RV2) were treated with (A) 2μm thapsigargin for 24 hrs, (B) 4μm calcimycin for 24 hrs, (C) serum starved for 24 hrs, or (D) exposed to 0.33 mM H$_2$O$_2$ for 4 hrs. Cell viability was measured using the XTT assay. Rat Hax-1 v1 protected H$_2$O$_2$ treated cells from cell death, as evidenced by the ~1.4 fold increase in cell viability compared to control cells (n=3, t-test, p<0.001, error bars show SEM), but failed to protect thapsigargin and calcimycin treated or serum starved cells. Conversely, rat Hax-1 v2 decreased cell viability of H$_2$O$_2$ treated cells by ~0.8 fold compared to control cells (n=3, t-test, p<0.04, error bars show SEM), but not of thapsigargin or calcimycin treated or serum starved cells. (E) HEK293 cells overexpressing RV1, RV2 or RV1 and RV2 and HV1, HV4 or HV1 and HV4 were treated with 0.33 mM H$_2$O$_2$ for 4 hrs, as in D, followed by evaluation of cell viability using the XTT assay. Similarly to the rat variants, human v1 and v4 decreased and exacerbated cell death of H$_2$O$_2$ treated cells by 20% and 10%, respectively, compared to control cells. Importantly, co-expression of rat v1 and v2 or human v1 and v4 counteracted the anti-apoptotic effect of v1 and the pro-death effect of v2/v4, resulting to cell viability levels similar to those observed in control cells expressing empty vectors (n=3, t-test, ** p<0.01, *** p<0.03, error bars show SEM).
We then used FACS analysis to examine how ectopic expression of the rat Hax-1 variants 1 and 2 and human Hax-1 variants 1 and 4 affected apoptosis of HEK293 cells following exposure to H$_2$O$_2$. We performed measurements of Annexin V PE and 7ADD at 4 and 24 hrs post-treatment to capture early and late cell responses. Approximately 90% of HEK293 cells expressing rat v1, human v1 or control empty vector were alive at 4 hrs following H$_2$O$_2$ treatment (Fig. 2.4A, and Fig. 2.5). Interestingly, only ~80% of HEK293 cells expressing either rat v2 or human v4 were alive at the same time point (Fig. 2.4A and Fig. 2.5). We then measured the levels of Annexin V PE and 7ADD 24 hrs post-treatment with H$_2$O$_2$. Approximately 80% of HEK293 cells expressing rat or human v1 remained alive at 24 hrs compared to 50% of control cells, demonstrating the protective effects of rat and human v1 (Fig. 2.4B). In contrast, <40% of cells expressing rat v2 or human v4 were alive at 24 hrs, indicating the pro-death effects of rat v2 and human v4 (Fig. 2.4B). Similar measurements in HEK293 cells co-expressing equivalent amounts of rat v1 and v2 or human v1 and v4 (Fig. 2.4) demonstrated that ~90% of cells were alive at 4 hrs, while 50% of cells remained alive at 24 hrs, similar to control cells expressing empty vectors at the same time points (Fig. 2.4A-B, and Fig. 2.5). Taken together, these results demonstrate that rat and human v1 promote cell survival under specific stress conditions (i.e. oxidative stress) by diminishing apoptosis, whereas rat v2 and human v4 exacerbate cell death under the same stress conditions by increasing apoptosis. Interestingly, co-expression of anti-apoptotic v1 and pro-death v2/v4 counteracts their individual effects, resulting in apoptotic levels similar to those of control cells.
Figure 2.4: Effects of Hax-1 variants on early and late apoptosis following treatment with H$_2$O$_2$. (A) HEK293 cells overexpressing RV1, HV1, RV2, HV4, RV1 and RV2 or HV1 and HV4 were treated with 0.33 mM H$_2$O$_2$ for 4 hrs. Apoptosis levels were evaluated by FACS analysis via staining with Annexin V PE and 7AAD 4 hours following treatment; only fluorescing cells were included in the analysis, to ensure that transfected cells were used. Data is presented as the percent of cells tested that were still alive; in other words cells that are negative for both Annexin V and 7AAD. Cells expressing RV1, HV1, RV1 and RV2 or HV1 and HV4 showed similar levels of apoptosis to those of control cells transduced with empty vector(s). In contrast, cells expressing RV2 or HV4 exhibited a 10% increase in apoptosis levels relative to control cells (n=3, t-test, p<0.13, error bars show SEM). (B) HEK293 cells were transfected and treated as in (A), but were incubated for an additional 24 hrs following H$_2$O$_2$ treatment. Cells expressing RV1 or HV1 showed a ~30% decrease in apoptosis levels compared to control cells expressing empty vector (n=3, t-test, p<0.01, error bars show SEM). In contrast, cells expressing RV2 or HV4 exhibited a ~10% increase in apoptosis levels relative to control cells (n=3, t-test, p<0.03, error bars show SEM). Importantly, cells co-expressing RV1 and RV2 or HV1 and HV4 showed similar levels of apoptosis to control cells, indicating that co-expression of v1 and v2/v4 eliminated their protective and pro-death effects, respectively.
Figure 2.5: FACS scatter plots of HEK293 cells expressing different Hax-1 variants or combinations thereof following treatment with H2O2. FACS scatter plots supplement figure 2.4, and show representative distributions of live and dying HEK293 cells expressing empty vector(s), rat Hax-1 v1 (RV1), rat v2 (RV2), rat v1 and v2 (RV1+RV2), human v1 (HV1), human v4 (HV4), or human v1 and v4 (HV1+HV4), 4 hours (A) and 24 hours (B) after H2O2 treatment. The top right quadrant includes the percentage (%) of 7ADD/Annexin-V PE positive cells, which are undergoing late apoptosis, while the bottom left quadrant includes the percentage (%) of 7ADD/Annexin-V PE negative cells, which are healthy and alive. The lower right quadrant shows Annexin V positive cells, which are early in the apoptotic process, and the upper left quadrant shows necrotic cells, which are only 7AAD positive.
The Hax-1 variants can form homotypic and heterotypic dimers with high affinity

To study the molecular mechanism through which Hax-1 v1 and v2/v4 counteract each other’s effect in regulating cell apoptosis, we examined their ability to form homotypic and heterotypic dimers, a property previously described for other members of the Bcl2 family (55,56). We generated full length recombinant rat Hax-1 v1 and v2 as GST- and His-tagged proteins (Fig. 2.6A), and performed a pull down assay (Fig 2.6B). Notably, both v1 and v2 were able to specifically and efficiently form homo- and hetero-dimers. To quantitate the binding affinity of these homo- and heterotypic interactions in real time, we performed surface plasmon resonance using a Biacore 3000 instrument (Fig 2.6 C and D). V1 formed homodimers with a Kd of ~4 nM, and heterodimers with v2 with a Kd of ~97 nM. Although the binding affinity of the v1 homodimer is ~25 fold higher than the binding affinity of the v1/v2 heterodimer, both interactions are strong, and most likely of physiological significance.

We were unable to efficiently capture Hax-1 v2 to CM5 or NTA chips as GST- or His-fusion proteins respectively, therefore kinetic measurements for the v2 homodimer were not feasible.
Figure 2.6: Hax-1 variants can form homotypic and heterotypic dimers. (A) Coomassie blue stained gel showing bacterially expressed rat Hax-1 v1 and v2 produced as GST- and His-tagged proteins. (B) GST-pull down assays were performed using equivalent amounts of control GST-protein, GST-Hax-1 v1 or GST-Hax-1 v2 bound to glutathione matrices, and His-Hax-1 v1 or His-Hax-1 v2. Retention of His-Hax-1 v1 or His-Hax-1 v2 was determined by immunoblot analysis using antibodies to the His-tag. GST-Hax-1 v1, but not control GST-protein, precipitated specifically and efficiently both His-Hax-1 v1 and His-Hax-1 v2. Similarly, GST-Hax-1 v2 retained specifically and efficiently His-Hax-1 v2. (C) Evaluation of the binding affinity of the Hax-1 v1 homodimer in real time using surface plasmon resonance and a Biacore 3000 instrument. GST-Hax-1 v1 was used as a ligand, and His-Hax-1 v1 was used as analyte at five different concentrations, ranging from 0-400 nM. A Kd of ~4 nM was calculated with a chi²=0.2 for fitted and obtained sensograms. (D) Evaluation of the binding affinity of the Hax-1 v1/v2 heterodimer in real time using surface plasmon resonance and a Biacore 3000 instrument. GST-Hax-1 v1 was used as ligand, and His-Hax-1 v2 was used as analyte at five different concentrations, ranging from 0-200 nM. A Kd of ~97 nM was calculated with a chi²=0.06 for fitted and obtained sensograms.
The COOH-terminus of Hax-1 supports its ability to homo- and hetero-dimerize

To determine the minimal region that supports the formation of Hax-1 homo- and hetero-dimers, we generated a series of deletion constructs and tested their ability to interact in the yeast two-hybrid system. We used full length rat Hax-1 v1 as bait, and three consecutive deletion constructs, referred to as A-C, as preys, containing amino acids 1-43, 35-102 and 97-278, respectively, and spanning the entire length of v1 (Fig. 2.7A). We also generated prey construct D containing amino acid residues 79-106, which are unique to v7. Prey construct C, including amino acids 97-278 interacted specifically and efficiently with full length v1 under high stringency conditions in the yeast system (Fig. 2.7B). Further deletion analysis of fragment C (aa 97-278) and generation of constructs C1 (aa 97-193) and C2 (aa 189-278) demonstrated that both C1 and C2 were able to support binding to full length v1, however C1 did so more efficiently and to the same extent as construct C. Additional deletion analysis and generation of constructs C3 (aa 97-148), C4 (aa 127-202), C5 (aa 188-224) and C6 (aa 225-278) resulted in complete abolishment of binding to full length v1 (Fig. 2.7B).

We then tested the ability of constructs C (aa 97-278), C1 (aa 97-193) and C2 (aa 189-278) to directly bind to full length Hax-1 v1 in a pull-down assay. We therefore generated recombinant GST-Hax-1 v1, which was immobilized to glutathione matrices and allowed to incubate with equivalent amounts of His-tagged C, C1 or C2 proteins (Fig. 2.7C). We observed that His-C, His-C1 and His-C2 proteins were able to specifically and directly bind to GST-Hax-1 v1, but not control GST-protein (Fig. 2.7D). Similarly to the yeast two-hybrid data, we consistently observed that His-C and His-C1 were retained more efficiently by GST-Hax-1 v1 compared to His-C2. Thus, it appears
that the C1 region containing residues 97-193 is the minimal site that mediates the ability of Hax-1 to form homotypic and heterotypic dimers, while the C2 region containing amino acids 189-278 may further contribute to the homo- or hetero-dimerization capability of Hax-1.

Notably, the COOH-terminal region of Hax-1 (aa 97-278) that also contains the homo- and hetero-dimerization site as our experiments indicated, is shared by all known Hax-1 variants and supports binding to a number of other proteins, as it was previously reported, including phospholamban, SERCA2, HtrA2, PARL, caspase 9, and HS1 (10).
Figure 2.7: Identification of the minimal binding site that supports the formation of Hax-1 homodimers and heterodimers. (A) Schematic representation of the Hax-1 bait and prey clones that were used in the yeast two-hybrid screen denoting the amino acid residues that were included in each construct. (B) Prey clones C (aa 97-278), C1 (aa 97-193) and C2 (aa 189-278) were able to support binding to bait construct Hax-1 v1, although to different extents, with constructs C and C1 exhibiting similar and higher affinity than construct C2. (C) Coomassie blue stained gel showing bacterially expressed GST-Hax-1 v1 and His-Hax-1 clone C, His-Hax-1 clone C1 and His-Hax-1 clone C2 proteins. (D) GST-pull down assays were performed using equivalent amounts of control GST-protein and GST-Hax-1 v1 bound to glutathione matrices, and His-Hax-1 clone C, His-Hax-1 clone C1 or His-Hax-1 clone C2 proteins. Retention of His-tagged proteins was examined by immunoblot analysis using antibodies to the His-tag. GST-Hax-1 v1, but not control GST-protein, precipitated specifically all three His-Hax-1 proteins, although with different efficiencies.
Overexpression of anti-apoptotic or pro-death Hax-1 variants fails to modulate the levels of intracellular ROS, but alters the cytoplasmic levels of cytochrome C following H$_2$O$_2$ treatment

We then investigated whether the anti-apoptotic effects of rat and human v1 and the pro-death effects of rat v2 and human v4 are due to their ability to regulate intracellular ROS levels. HEK293 cells overexpressing rat Hax-1 v1, v2, or v1 and v2, and human Hax-1 v1, v4 or v1 and v4 were treated with H$_2$O$_2$, and the levels of intracellular ROS were measured 1 and 4 hrs post-treatment. As expected, all cell populations exposed to H$_2$O$_2$ exhibited significantly higher levels of intracellular ROS compared to untreated HEK293 cells expressing empty vector(s) (Fig. 2.8A). Interestingly though, we did not observe statistically significant differences in the levels of intracellular ROS at 1hr (Fig. 2.8A) or 4 hrs (not shown) following H$_2$O$_2$ treatment, among cell groups overexpressing v1 or v2/v4 or cell groups co-expressing v1 and v2/v4, compared to control cells containing empty vector(s). Therefore, we then examined if the opposing effects of v1 and v2/v4 are exerted via regulation of the levels of cytosolic cytochrome C, which we measured in the aforementioned HEK293 cell groups (Fig. 2.8B). We initially performed a time course (0.5-24 hrs; not shown) to determine the time point of maximal cytochrome C release, which we found it to be 2 hours post treatment. HEK293 cells overexpressing rat v2 or human v4 exhibited significantly higher levels of cytosolic cytochrome C (Fig 2.8B; ~1.4 fold increase), compared to cells overexpressing rat or human v1, rat v1 and v2, human v1 and v4 or empty vector(s), as evidenced by densitometry of the respective immunoreactive bands.
Taken together, these findings indicate that the antagonistic roles of v1 and v2/v4 are not exerted via modulation of intracellular ROS levels, but through regulation of cytochrome C release.
**Figure 2.8:** *Measurements of intracellular ROS and cytoplasmic cytochrome C levels in HEK293 cells expressing distinct Hax-1 variants or combinations thereof following H$_2$O$_2$ treatment.* (A) HEK293 cells overexpressing rat Hax-1 v1 (RV1), v2 (RV2), and RV1 and RV2 or human v1 (HV1), v4 (HV4), and HV1 and HV4 were treated with 0.33 mM H$_2$O$_2$ for 1 and 4 hrs (the 4-hour data is not shown, since it is statistically equivalent to the 1-hour data), and intracellular ROS levels were measured using the Oxiselect ROS assay. Intracellular ROS levels were significantly higher in cells treated with H$_2$O$_2$ compared to untreated control cells (n=3, t-test, p<0.03, error bars show SEM). However, there was no statistically significant difference in intracellular ROS levels between treated cells expressing empty vector(s) and treated cells expressing the different Hax-1 variants or combinations thereof (n=3, t-test, p> 0.3, error bars show SEM). These results indicate that Hax-1 v1 and v2/v4 do not regulate H$_2$O$_2$ induced apoptosis by modulation of intracellular ROS levels. (B) HEK293 cells overexpressing RV1, RV2, and RV1 and RV2, or HV1, HV4, and HV1 and HV4 or empty vector(s) were treated with 0.33 mM H$_2$O$_2$ for 2 hours. Cell groups were then subjected to subcellular fractionation to collect the cytoplasmic fractions using the Abcam Cytochrome C release kit, which were analyzed by immunoblotting using antibodies to cytochrome C. As expected, untreated cells contain undetectable levels of cytoplasmic cytochrome C, while treated cells expressing empty vector(s), RV1, HV1, RV1 and RV2, or HV1 and HV4 have similar, yet low, amounts of cytoplasmic cytochrome C. In contrast, treated cells expressing RV2 or HV4 show notably higher levels of cytosolic cytochrome C (~1.4 fold increase compared to control cells), indicating its release from the mitochondria and the initiation of apoptosis. GAPDH served as loading control.
Figure 2.8: Measurements of intracellular ROS and cytoplasmic cytochrome C levels in HEK293 cells expressing distinct Hax-1 variants or combinations thereof following H$_2$O$_2$ treatment.
2.4 Discussion: Implications of antagonistic roles of Hax-1 variants

Hax-1 v1 is a ubiquitously expressed protein that has been extensively studied in humans and rodents with established anti-apoptotic activity (10,19). Contrary to the mouse gene, the human and rat HAX1 genes are heavily spliced giving rise to at least seven distinct variants that primarily differ in the NH2-terminus (1). In the current study, we examined the expression profile of Hax-1 variants in normal and stressed rat myocardium. We observed a significant increase in the transcript and protein levels of Hax-1 v2 twelve weeks post-induction of myocardial infarction. Consistent with this, rat v2 and its human homologue v4 exacerbated cell death under conditions that led to oxidative stress, and abrogated the anti-apoptotic effect of rat and human v1. Rat v2 and human v4 are structurally similar to the rat and human v1. They only differ in the retention of exon 2, which is present in all known v1 proteins, but is partly spliced out in the rat v2 and human v4 isoforms (1). The spliced-out portion of exon 2 encodes 61 amino acids in rats and 48 amino acids in humans, comprising the predicted BH1 and BH2 domains and flanking sequence. It therefore appears that the BH1 and BH2 domains present in Hax-1 v1 confer its anti-apoptotic activity, which is consistent with earlier findings for other members of the BCL2 family of apoptotic regulators (57-59).

Previous studies have demonstrated that Hax-1 v1 can interact specifically and directly with several proteins that are intimately involved in the regulation of apoptosis via distinct mechanisms. These include phospholamban (PLN) (7) and SERCA (14), which are major modulators of Ca^{2+} cycling, mitochondrial proteases Presenilins-Associated Rhomboid-Like protein (PARL), and High temperature regulated A2 (HtrA2) (18), and cytoplasmic pro-caspase 9 (11,18). Consistent with these findings, it has been
postulated that direct binding of Hax-1 v1 to PLN and SERCA modulates Ca\textsuperscript{2+} homeostasis (14,17,60). Alternatively, direct binding of Hax-1 v1 to HtrA2 and PARL facilitates the PARL-mediated activation of HtrA2 (18). Activated HtrA2 prevents the accumulation of pro-apoptotic BAX in the mitochondrial outer membrane, thus inhibiting mitochondrial pore opening and cytochrome C release (18). Moreover, direct binding of Hax-1 v1 to initiator pro-caspase 9 precludes its activation, leading to failure of activation of death pro-caspase 3 (6,7).

Our studies demonstrate that Hax-1 proteins can also form homotypic and heterotypic dimers with affinities in the low nanomolar (nM) range, suggesting that these are strong physiological interactions that take place within the cell. Whether Hax-1 v1 interacts with the battery of identified binding partners as a monomer, a homodimer or a heterodimer is currently unknown. Interestingly though, nearly all the known binding interactions of Hax-1 v1, including the formation of homo- and hetero-dimers, are mediated by amino acid residues present in the COOH-terminal region of the molecule (amino acids 97-278). This finding has two important implications. First, there is likely competition among Hax-1 v1’s partners for binding, which may be regulated by their relative expression levels, activation status, or subcellular distribution, given that v1 may localize to and possibly oscillate between the sarco/endoplasmic reticulum and the mitochondria (19,40), and secondly, the COOH-terminal region that contains all currently identified binding sites is shared by all known Hax-1 isoforms, indicating that other Hax-1 variants with potentially distinct, if not opposing, roles than v1 (e.g. v2/v4) may directly interact with the same proteins, and thus antagonize v1’s anti-apoptotic activity.
It is therefore possible that Hax-1 v2/v4 may directly bind to HtrA2 and PARL, leading to their inactivation.

Conversely, Hax-1 homodimers or heterodimers may act independently of other Hax-1 binding partners. Thus, homodimerization may be required for v1 to exert its anti-apoptotic effect, and for v2/v4 to potentiate cell death following insult. Alternatively, heterodimerization may neutralize the individual effects of v1 or v2/v4 via sequestration. Thus, overexpression of rat v2 or human v4 may promote cytochrome C release following $\mathrm{H}_2\mathrm{O}_2$ treatment by heterodimerizing and sequestering endogenous v1, thus preventing it from facilitating PARL-mediated activation of HtrA2 and BAX removal from the outer mitochondrial membrane (18).

It therefore becomes apparent that there is a high degree of previously unforeseen complexity that characterizes the homotypic, heterotypic and allotopic binding interactions of the Hax-1 proteins, which may depend on their ratio, subcellular localization, as well as the presence and type of stress exerted to the cell.

The antagonistic roles of Hax-1 v1 and v2/v4 are reminiscent of the opposing roles of the BCL-X splice variants, BCL-Xl and BCL-Xs (61,62). BCL-Xl binds to and sequesters BAX and BAK, inhibiting them from forming pores in the outer mitochondrial membrane, and thus preventing cytochrome C release (58,61). BCL-Xs, similarly to Hax-1 v2/v4, lacks the BH1 and BH2 domains present in BCL-Xl and Hax-1 v1, and promotes cell death in the presence of a stress stimulus (63). Although the exact mechanism of action of BCL-Xs is still unknown, it has been shown that it can bind to and inhibit or sequester anti-apoptotic proteins BCL-Xl and BCL2 (57,62,64-66).
Notably, the pro-apoptotic effect of BCL-Xs is counteracted by overexpression of BCL2 or BCL-XI (55), similarly to Hax-1 v2/v4 and v1.

Several anti-apoptotic BCL2 family members, such as BCL2 and BCL-XI have been shown to prevent the accumulation of ROS within cells, thus protecting against ROS-induced apoptosis (67,68). Interestingly, cells overexpressing Hax-1 v1 failed to reduce the levels of intracellular ROS following H$_2$O$_2$ treatment, compared to control cells, but exhibited reduced levels of cytoplasmic cytochrome C. Conversely, cells overexpressing Hax-1 v2/v4 failed to increase the levels of intracellular ROS, but displayed increased levels of cytoplasmic cytochrome C. These findings indicate that the anti-apoptotic and pro-death effects of Hax-1 proteins are not mediated via regulation of intracellular ROS levels, but through modulation of cytochrome C release, possibly via the HtrA2/PARL pathway (18,69).

Taken together, our findings demonstrate that Hax-1 comprises a family of apoptotic regulators in response to oxidative stress, with antagonistic roles. The ability of Hax-1 proteins to form homo- and hetero-dimers and to interact with diverse proteins involved in the regulation of cell fate indicates that modulation of cell survival or death via the Hax-1 family is highly complex. Further work is required to delineate the precise molecular mechanisms via which Hax-1 proteins may promote cell survival or apoptosis, and to establish how their ratio, subcellular distribution, and preferential homotypic, heterotypic or allotypic binding interactions contribute to the determination of cell fate under stress conditions.
Chapter 3: Challenges in studying Hax-1 in vivo.

3.1 Introduction

As previously discussed, most of the published work on Hax-1 focuses on variant 1. Not only is this because Hax-1 variant 1 is the prototypical variant, but also because variant 1 is a unique size, distinguishable from the other known variants, and is proven to be recognized by all commercially available Hax-1 antibodies, thus facilitating its study. While this has led to a better understanding of the function(s) of Hax-1 variant 1, recent findings on the antagonistic roles of some of the Hax-1 variants and their ability to dimerize suggest that studying variant 1 alone only gives part of the story.

As a result of the difficulty distinguishing the Hax-1 variants in vivo, there is little or no knowledge of the dimerization status of Hax-1 and its interaction with other known binding partners in vivo. Knowing the dimerization status of Hax-1 under normal and stress conditions, the relative ratios of the Hax-1 variants present, and the circumstances under which Hax-1 interacts with its other binding partners would be key to elucidating Hax-1’s mechanism of action. This would not only help us to better understand the anti-apoptotic role of variant 1, but could also lead to the discovery of how the pro-apoptotic Hax-1 variant(s) function. We hypothesize that the subcellular distribution, relative ratios, and dimerization state of Hax-1 determines cell fate following insult.

Herein, an attempt was made to generate antibodies specific for several rat Hax-1 variants, thus making it easier to study specific variants in vivo. Variant specific antibodies would facilitate the study of the subcellular localization and abundance of specific variants under both normal and stress conditions and to identify co-localization
and interaction between specific variants. Such antibodies would also allow for the identification of the dimerization state of select Hax-1 variants and the interaction of Hax-1 with other binding partners under varying conditions. These experiments would be made possible by the ability to differentially label, immunoprecipitate, or immunoblot for specific endogenous Hax-1 variants in vivo, none of which are currently possible.

Immunoprecipitations of Hax-1 from rat cardiac tissue before and after myocardial infarction were also attempted, in order to gain a better understanding of the mechanism of action of Hax-1. By performing immunoprecipitations of Hax-1 under normal and disease conditions, the binding partners of Hax-1 could be identified in each state. This would provide valuable information about the dynamics of Hax-1’s interactions with other proteins as well as providing insight into a molecular mechanism of action.

3.2 Experimental procedures

Generation of variant specific Hax-1 antibodies

Unique regions of the rat Hax-1 variant proteins were identified by amino acid sequence alignment, and were all identified to be from either exon-exon junctions or exons only present in specific variants. These unique peptide sequences were then sent to Open Biosystems (Open Biosystems / Thermo Fisher Scientific, Huntsville, AL, USA) and analyzed for antigenicity using their proprietary prediction software. Three peptide regions were selected as being highly antigenic and good candidates for immunization into rabbits to generate antibodies: RGFFGFPGR, DLFRGFFGFPGR, and GPERRKQWGSGKEDREQ. These three peptides were recombinantly produced, used
to immunize two rabbits each, and crude serum was collected from the rabbits after 90
days. This work was done in house by Open Biosystems. Upon receipt of the frozen
serum samples, the Pierce AminoLink Kit was used to purify the serum antibodies raised
against the Hax-1 peptides, following the manufacturer’s protocol (Thermo Fisher
Scientific, Rockford, IL, USA).

**Myocardial Infarction (MI)**

Frozen lyophilized heart tissue from adult Sprague Dawley rats was generously
donated by Dr. William Stanley (University of Maryland, School of Medicine). Heart
failure was induced by constriction of the left coronary artery to simulate myocardial
infarction (MI), as previously described (45,46). Control sham animals underwent the
same surgery without artery ligation. Heart tissue was collected from both groups 12
weeks after surgery.

**Production of recombinant Hax-1 proteins**

N-terminally 6x His tagged rat Hax-1 variants 1, 2, and 4 were recombinantly
produced as in section 2.2.

**Dot Blots**

10, 25, and 50 ng of purified peptide, in PBS, was pipetted onto nitrocellulose membrane
strips in a single “dot” for each concentration. Membranes were then air-dried for 30
minutes, and then a standard immunoblotting procedure was followed, as previously
described (19). Our purified Hax-1 antibodies were used at a final concentration of 600
ng/ml, followed by donkey anti-rabbit secondary antibody (1:2500 dilution, Jackson
Imunoresearch, West Grove PA, USA). Dot blots were then developed using an alkaline phosphatase based chemiluminescent system (Applied Biosystems, Foster City CA, USA).

**Western blotting**

Refer to section 2.2 for immunoblotting procedure. Western blots were either run using tissue homogenates prepared from flash frozen isolated adult Sprague Dawley rat tissue, or 10 µg of recombinant purified Hax-1 proteins, as described above. Homemade Hax-1 antibodies were used at a concentration of 600 ng/ml or a 1/1000 dilution of BD anti-Hax-1 primary antibody (BD Biosciences, Franklin Lakes NJ, USA) were used for immunoblotting, followed by donkey anti-rabbit secondary antibody (1:2500 dilution, Jackson Imunoresearch, West Grove PA, USA). Alpha tubulin (1:1000 dilution, Sigma Aldrich, St. Louis MO, USA) primary antibody was used as a load control for all blots.

**Hax-1 Immunoprecipitations**

25 mg of lyophilized rat heart tissue was homogenized in 200 µl NP40 buffer containing 10 mM NaPO₄, 2 mM EDTA, 10 mM NaN₃, 0.9% NaCl, 2% NP40 in the presence of a protease inhibitor cocktail (Roche, Indianapolis IN, USA, product number 11697498001) using a tissue grinder (VWR, Radnor PA, USA, model 47747-366). 1 mg of total homogenate was pre-cleared by incubating with 50 ul of Protein A/G agarose bead slurry (Thermo Fisher Scientific, Rockford, IL, USA, product number 20421) in 500 ul total volume PBS for 12 hours at 4°C. Likewise, 5 µg of Santa Cruz biotechnology Hax-1 primary antibody (Santa Cruz Biotechnology, Dallas TX, USA, product number sc-166845) was incubated with 50 µl of protein A/G agarose bead slurry (Thermo Fisher
Scientific, Rockford, IL, USA, product number 20421) in 500 µl total volume PBS for 12 hours at 4°C, in order to coat the beads with the Hax-1 antibody. After pre-clearing, the homogenate samples were spun at 2500 x g for 3 minutes and the supernatants were added to the Hax-1 antibody plus bead mixes and incubated at 4°C for 16 hours. The samples were then spun for 3 minutes at 2500 x g, supernatants removed, and washed with 500 µl cold PBS. This wash step was repeated 5 times. Elutions were performed in PBS with 0.5% RapiGest SF Surfactant (Waters corporation, Milford, MA, USA, product number 186002122), collected in 500 µl volumes, and mixed with 50 µl neutralization buffer (1 M Tris pH 8) immediately after collection. The entire procedure described above was later repeated using Dynabeads Protein A (Life technologies, Grand Island, NY, USA, product number 10001D), instead of the Protein A/G agarose beads (Thermo Fisher Scientific, Rockford, IL, USA, product number 20421).

3.3 Results: Specificity of custom Hax-1 antibodies and in vivo Hax-1 immunoprecipitations

Specificity of rat homemade Hax-1 antibodies

The affinities of the purified antibodies to the antigenic peptides were checked by dot blot. All 3 homemade antibodies were tested against all 3 peptides, to check for specificity. Antibody number 1 was raised against peptide RGFFGFPGR, antibody 2 was raised against peptide DLFRGFFGFPGR, and antibody 3 was raised against peptide GPERRKQWGSGKEDREQ. As can be seen by sequence similarity, antibodies 1 and 2 should recognize both peptides RGFFGFPGR and DLFRGFFGFPGR, while antibody 3 should be specific to GPERRKQWGSGKEDREQ. Antibodies 1 and 2 should recognize rat Hax-1 variants 1, 2, 8, and 10 which are 31.5, 24.5, 27.5, and 14.5 kDa,
respectively, and would be uniquely identifiable by size via western blot. Antibody 3 should only recognize rat Hax-1 variants 3 and 4 which are 32 and 25.5 kDa, respectively. As predicted, antibodies 1 and 2 were able to recognize each other’s peptides, RGFFGFPGPR and DLFRGFFGFPGR, but not GPERRKQWGSGKEDREQ, in a dot blot using as little as 10 ng of peptide. Antibody 3 was specific for peptide GPERRKQWGSGKEDREQ via dot blot with 10 ng of peptide.

The three homemade Hax-1 antibodies were then tested for specificity against recombinantly produced N-terminally 6x His tagged Hax-1 variants 1, 2, and 4 via western blot. The blots were run in triplicate, each probed with a different homemade Hax-1 antibody. Antibodies 2 and 3 were unable to detect any of the purified protein, while antibody 1 was able to recognize both Hax-1 variants 1 and 2.

Antibodies were again tested via western blot using samples of rat heart, tibialis anterior (TA), skin, lung, kidney, liver, and brain. Blots were run in quadruplet, and were probed with either BD anti-Hax-1 primary antibody, homemade anti-Hax-1 antibody 1, 2, or 3. Band patterns of the immunoblots were then compared in order to try to determine the Hax-1 variant specificity of the homemade antibodies as well as the tissue expression pattern of the different rat Hx-1 variants. The BD anti-Hax-1 antibody was used as a positive control. The BD Hax-1 antibody produced bands of ~35 and ~25 kDa, representing rat Hax-1 variants 1 and 2 respectively, in all samples tested. Unfortunately, all three homemade Hax-1 antibodies failed to identify any specific Hax-1 variants. Antibody 1 produced ~5 immunoreactive bands from the heart sample, all of which were larger than 50 kDa. We were unable to determine if these bands represented Hax-1 dimers, post-translational modifications, uncharacterized variants, or non-specific
antibody binding. Furthermore, no immunoreactive bands were seen with antibody 1 in the TA, skin, lung, kidney, liver, or brain samples. Antibodies 2 and 3 both produced a ‘ladder’ pattern, containing at least 10 different immunoreactive bands for every tissue tested. Again, none of these bands were able to be identified as specific variants as none corresponded to sizes of known rat Hax-1 variants.

Hax-1 immunoprecipitations were performed with rat heart homogenates from both normal animals and those having undergone MI using the commercially available Hax-1 antibody from BD Biosciences. Unfortunately, successful immunoprecipitations were not able to be performed due to nonspecific binding of several proteins in the homogenate samples, including Hax-1, to the beads needed for precipitation. Several different pre-clearing protocols were attempted, with increasing incubation times, increased concentrations of detergents in the buffer, reduced temperature, and different types of beads to eliminate this nonspecific binding. While the levels of proteins bound directly to the beads, in the absence or presence of antibody, was reduced it could not be sufficiently eliminated. These immunoprecipitations were originally intended to be performed using our homemade Hax-1 variant specific antibodies, but that was found to be impossible. Instead, the BD Biosciences Hax-1 antibody was used, which would pull down several different Hax-1 variants.

3.4 Discussion: Challenges in distinguishing the Hax-1 variants

Among the Hax-1 variants, there are very few, if any, unique peptide regions specific to single variants. Accordingly, regions shared by only a few variants were chosen as antigens for antibody design. These unique regions are limited to exon
junctions, as the splicing of Hax-1 mostly involves the retention or skipping of exons 1 and 2 or introns 1 and 2. Among the exon junctions that are unique to only a few Hax-1 variants, most were not antigenic enough for antibody production, leaving only two regions of the Hax-1 protein as viable antibody targets. One region, the junction of exon 1 and exon 2a which is only found in rat Hax-1 variants 1, 2, 8, and 10, was represented by two different peptides: RGFFGFPGPR, and DLFRGFFGFPGPR (used to raise homemade antibodies 1 and 2, respectively). The other region is the junction of intron 1a and exon 2a and was represented by the peptide GPERRKQWGSGKEDREQ (used to raise homemade antibody 3) and is only present in rat Hax-1 variants 3 and 4. As should be the case, all three homemade antibodies were able to recognize the peptides they were raised against, when tested via dot blot.

Interestingly, antibodies 2 and 3 were unable to detect purified rat Hax-1 variants 1, 2, or 4 proteins, while antibody 1 was able to recognize both Hax-1 variants 1 and 2. Homemade antibody 1 performed as expected, however it was anticipated that antibody 2 would also recognize purified rat Hax-1 variants 1 and 2 and that antibody 3 would recognize purified Hax-1 variant 4. The purified Hax-1 proteins used were 6x His tagged on the N-terminus, which may have interfered with antibody recognition as all three antibody targets are near the N-terminus. The recognition sequence for antibody 1 begins at the eighth amino acid from the N-terminus, while the recognition sequence for antibodies 2 and 3 begin at amino acids 3 and 4, respectively. Steric hindrance or altered folding of the recombinant His tagged Hax-1 proteins may have blocked the binding of homemade antibodies 2 and 3, since their recognition sites are so close to the His tag.
Homemade Hax-1 antibody 1 produced 5 immunoreactive bands for rat heart homogenate via western blot, but failed to identify Hax-1 in TA, skin, lung, kidney, liver, and brain samples. All of the bands produced from rat heart were greater than 50 kDa, and are thus larger than any known Hax-1 variants. These bands may represent Hax-1 dimers that were not disrupted by the denaturing conditions of the gel, since the dimerization state of Hax-1 is thought to be an important mechanism for regulating apoptosis in the heart. It is unclear why antibody 1 failed to recognize Hax-1 in any of the other tissue samples, while the control BD Hax-1 antibody identified variants 1 and 2 in every sample tested. Although the results generated from homemade antibody 1 are inconclusive, further optimization of binding and blotting procedures may result in valuable information specific to variants 1, 2, 8, and 10.

While homemade antibodies 2 and 3 failed to recognize purified His tagged Hax-1 protein, they generated a ‘ladder’ pattern of bands via western blot in all tissues tested. This is most likely nonspecific binding, as it is unlikely that there are dozens of unknown Hax-1 variants containing these specific peptide regions. Altering conditions of the western blots was unable to improve specificity. Due to the failure of antibodies 2 and 3 to recognize purified Hax-1 protein and their apparent nonspecific interactions with other proteins in tissue homogenates, they are not useful for the study of the Hax-1.

Ideally, the Hax-1 immunoprecipitations could have been performed with our homemade Hax-1 variant specific antibodies, but that was not possible. The BD Hax-1 antibody was used instead, which reliably binds to rat Hax-1 variants 1 and 2. While the pre-clearing step of an immunoprecipitation experiment is designed to eliminate nonspecific binding interactions, we were unable to overcome this problem. With both
the protein A/G agarose beads and Dynabeads Protein A, Hax-1 was pulled down in the absence of antibody. Increasing the bead volume or pre-clearing time resulted in near compete removal of Hax-1 from the sample homogenates. Other proteins were also nonspecifically pulled down by the beads, but it is unclear whether they interacted directly with the beads or were bound to Hax-1. The main goal of performing these Hax-1 immunoprecipitations was to determine the binding partners of Hax-1 under both normal and disease states and to use this information to piece together Hax-1’s molecular mechanism of action. The number of proteins pulled down by the beads alone was significant, and excluding them from the study could have excluded important binding partners of Hax-1, since it was unclear whether they were pulled down due to nonspecific interaction with the beads or because they were bound to Hax-1. Because this critical distinction could not be made, and because the pulldowns were not able to be ‘cleaned up’, they were of no use in determining the molecular mechanism of Hax-1. Determining the mechanism by which Hax-1 acts, and how the relative expression of the different variants and their dimerization state effects cell death is still critical to our understanding of apoptosis. New tools such as Hax-1 variant specific antibodies will make this work much easier and allow the use of new techniques not currently feasible for Hax-1.
Chapter 4: Conclusions and future directions for studying Hax-1

The HAXI gene is ubiquitously expressed in humans and rats and gives rise to at least 7 different splice variants, which mostly differ in their NH$_2$-terminus (1). The prototypical variant 1 has been most comprehensively studied and has been shown to have anti-apoptotic activities in response to certain insults (10,19). For the first time, we examined the expression profile of several different Hax-1 variants in adult rat myocardium before and after MI. While all Hax-1 transcripts studied had relatively similar expression levels in normal hearts, the transcript level of Hax-1 variant 2 was significantly up-regulated following MI. An increase of Hax-1 variant 2 was also seen at the protein level via western blot. This finding suggests that Hax-1 variant 2 plays a role in injury response in the rat heart.

Herein, we showed for the first time that Hax-1 comprises a family of apoptosis regulators with antagonistic roles. We showed that both rat Hax-1 variant 2 and human Hax-1 variant 4 (human Hax-1 variant 4 is homologous to rat Hax-1 variant 2) promote cell death after insult, and function in the opposite manner to variant 1. There are at least 7 characterized Hax-1 variants in human and rat, and several more that have yet to be described. The majority of these variants have unknown functions, and it can no longer be assumed that they function similarly to variant 1. In the rat, variant 2 differs from variant 1 only in that it lacks 61 amino acids near the N-terminus that comprise the predicted BH1 and BH2 domains. It may be premature to assign this region to the ability of Hax-1 variant 1 to resist apoptosis, as other factors may be involved such as subcellular localization or relative abundance of the variants. Rat Hax-1 variants 4 and 7
are also lacking the same 61 amino acid region as variant 2, suggesting that they may function in a similar pro-apoptotic manner. However, variant 4 contains an N-terminal nuclear localization sequence while variant 2 contains a mitochondrial targeting sequence and variant 6 does not contain any localization sequence. More studies are needed to determine what effect the subcellular localization of the Hax-1 variants has on their function. Further work is also needed to identify the role that other Hax-1 variants play in both the rat and human and how their sequences and domain structure may contribute to their function.

Accordingly, the research focus on Hax-1 should shift from the prototypical variant 1, to the dynamics of and interactions between the opposing Hax-1 variants. Our research also showed, for the first time, the ability of the Hax-1 variants to dimerize. It was shown that rat Hax-1 variants 1 and 2 can form both homo- and heterodimers. This was confirmed by both GST pulldown assays using purified recombinant Hax-1 proteins, and by surface plasmon resonance, yielding nM Kd’s for all interactions. In addition to the relative amounts and localization of the variants, their dimerization state must also play a role in regulating apoptosis. Accordingly, we hypothesize that the subcellular distribution and relative ratios of the Hax-1 variants determines which dimers will form, and ultimately determine cell fate. Our current hypothesis is that Hax-1 variants 1 and 2 in rat or 1 and 4 in human dimerize to inactivate or sequester one another. This is supported by our findings that co-expression of these variants in HEK293 cells abrogates both the protective effect of variant 1 and the pro-death effect of variants 2 and 4. The dimers formed by the Hax-1 variants may be regulated by their differential subcellular localization, making only the Hax-1 variants in close proximity able to dimerize.
Additionally, the dimerization state of Hax-1 could be controlled by the relative abundance of the variants, where Hax-1 dimers will form until the limiting variant is completely bound, leaving the variant expressed in excess free to interact with other proteins.

While the dimerization state of Hax-1 is likely an important factor in regulating apoptosis, it is currently not known the extent to which different variants can dimerize. After discovering the dimerization of the Hax-1 variants, we sought to characterize the binding region(s). To achieve this, we used yeast two hybrid and found that the dimerization site consists of amino acids 97-278, with amino acids 97-193 being the minimal region for high affinity dimerization. This region is shared by both rat Hax-1 variants 1 and 2, which were used in this study, but also by variants 3, 4, 5, 6, and their human homologues. The ability of both amino acid regions 97-193 and 97-278 to form dimers with full length Hax-1 was also confirmed via GST pull down using recombinantly produced proteins.

Using the yeast two hybrid system, we recently discovered that the minimal dimerization site, amino acids 97-193, binds to itself to form Hax-1 dimers rather than another location in the protein. We are currently working to confirm this interaction with recombinant proteins. This supports the notion that Hax-1 variants 1, 2, 3, 4, 5, and 6 may be able to dimerize, as they all share this region, and that Hax-1’s ability to dimerize is not affected by splicing events at the NH2-terminus. Not only does this finding imply that there is competition between the Hax-1 variants for dimerization, which is likely regulated by their relative expression levels or subcellular distribution, but also suggests
that there is competition between the Hax-1 variants and other binding partners, since the dimerization site contains nearly all other known binding sites in Hax-1 (Fig 4.1).
Fig 4.1: Binding sites in the Hax-1 protein. A schematic representation of rat Hax-1 variant 1 is shown with the predicted domains. Domain abbreviations are as follows: mitochondrial targeting sequence (MTS), acidic box (EE), Bcl-2 homology domains 1 and 2 (BH1 and BH2), Proline (P), Glutamic Acid (E), Serine (S), Threonine (T) rich motif, (PEST), predicted transmembrane (TM) domain, and Integrin β6 binding domain (β6); numbers on top indicate amino acid residues defining the domains. Known binding sites in the Hax-1 protein are schematically pictured as black lines, and labeled with their binding partner(s) and amino acid residue numbers (10,70). It should be noted that some binding sites have only been experimentally confirmed in rat or in human, but not both, but are all shown in relation to rat Hax-1 variant 1 for simplicity.
We are also currently working to express Hax-1 deletion constructs, lacking both
the full and minimal dimerization sites (amino acids 97-278 and 97-193, respectively) in
HEK293 cells. We will then seek to characterize what effect, if any, these truncated Hax-
1 variants have on apoptosis following H$_2$O$_2$ exposure. If Hax-1 variants lacking the
minimal dimerization site are able to confer the same apoptotic effect as their full length
counterparts, it will support our hypothesis that dimerization is the mechanism by which
the pro- and anti-apoptotic Hax-1 variants are regulated, but that dimerization is not
necessary for Hax-1 to either prevent or promote apoptosis. Myc tagged Hax-1 variants,
both full length and truncated, are also being overexpressed in HEK293 cells. The Myc
tag will allow us to immunoprecipitate the exogenous Hax-1 variants and identify which
endogenous Hax-1 variants are bound under what conditions. Concurrently, the
conditions under which Hax-1 interacts with its other binding partners could also be
identified. We hypothesize that dimerization sequesters the Hax-1 variants, leaving the
free Hax-1 able to interact with other proteins and confer its apoptotic function and will
test this hypothesis using the Myc tagged Hax-1 deletion constructs. This is supported by
our findings that the co-expression of rat Hax-1 variants 1 and 2 or human Hax-1 variants
1 and 4 results in the abrogation of the individual effects of both variants.

Since there is currently no practical way to distinguish different endogenous Hax-
1 variants in either the rat or human, future studies are somewhat limited. Further in vivo
studies of specific Hax-1 variants will require the use of tagged exogenous proteins, as
there are currently no commercially available Hax-1 antibodies that recognize only
specific variants. Using exogenous tagged Hax-1 proteins in vivo, we will be able to
better study the expression levels, localization, dimerization, and downstream effectors of Hax-1.

Further work is needed to characterize the expression patterns of the Hax-1 variants under normal conditions, after injury, and in disease state(s). The significant up-regulation of rat Hax-1 variant 2, at both the transcript and protein levels, in rat heart following MI has led us to discover its pro-apoptotic function. However, the mechanism by which Hax-1 variant 2 promotes cell death is still unknown. We showed that overexpressing the Hax-1 variant(s) in HEK293 cells had no effect on intracellular ROS levels following \( \text{H}_2\text{O}_2 \) treatment, suggesting that Hax-1 is not involved in the regulation of ROS and controls apoptosis in some other manner. We also showed an increase in cytochrome C release from the mitochondria in HEK293 cells overexpressing rat Hax-1 variant 2 or human variant 4, relative to controls and cells overexpressing variant 1 or both variants together, following \( \text{H}_2\text{O}_2 \) treatment. This finding suggests Hax-1’s involvement in the canonical intrinsic apoptosis pathway; however its exact mechanism has yet to be identified. We hypothesize that Hax-1 variant 1 activates Parl and HtrA2 in the mitochondrial intermembrane space, thereby inhibiting BAX from forming pores in the outer membrane and preventing cytochrome C release. The presence of variant 2 above a certain ‘threshold’ level may be sufficient to disrupt this mechanism and initiate apoptosis, but it is more likely that the ratio of the pro- to anti-apoptotic Hax-1 variants is more important than total levels. The binding sites for most of the interacting partners of Hax-1 are shared by both variants 1 and 2, including the binding sites for Parl and HtrA2, so it is possible that variant 2 can also directly interact with these proteins. Variant 2 may be directly sequestering Parl and HtrA2 or might be dimerizing with variant 1, to prevent
variant 1 from activating these anti-apoptotic processes. It seems that Hax-1 only regulates apoptosis in response to certain insults, and further work is necessary to fully understand what insults, injuries, or diseases Hax-1 is involved in and how the regulation and interaction of the Hax-1 variants control cell fate.


51. Peter W. Reed and Henry A. Lardy (1972) *Journal of Biological Chemistry* 247, 6970-6977


