Identification and Characterization of HAX1 Interacting proteins

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An Offering

At thy lotus feet

Zusammenfassung

Das “Kostman Gen” kodiert ein Protein, das in der Literatur als HAX-1 bezeichnet wird und mit dem HS-1 Protein X, ein mitochondriales 35kDA grosses intrazelluläres Protein, assoziiert ist. Bif-1, Teil der SH3 Domänenfamilie der Endophilin und Serpin b1a Gruppe der Ova-Serpin Superfamilie wurde mittels eines *Yeast-Two-Hybrid Screens*, als mit HAX-1 interagierendes Protein identifiziert.

Eine Überexpression von HAX-1 in E.coli war aufgrund der möglichen Präsenz von seltenen *Codons* und *Codon Bias* nicht möglich. Ferner war die Überexpression von HAX-1 in *Dictyostelium discoideum* aufgrund der gegebenen Instabilität dieses Proteins nicht zu erreichen.

Es konnte gezeigt werden, dass Bif-1 und HAX-1 sowohl in 293T als auch in Jurkat Zellen endogen miteinander interagieren. Und dass der C-Terminus von HAX-1, Aminosäuren 179 bis 234, in diese Bindung mit Bif-1 involviert ist. Im Cytoplasma konnte eine signifikante Menge von endogenem HAX-1 und Bif-1 nachgewiesen werden, die dort Co-Lokalisiert vorlagen. Eine *EBSS-Starvation* führte in HAX-1 defizienten Patientenfibroblasten durch Autophagie zum Zelltod. Ferner belegen die Daten, dass eine *EBSS-Starvation* in HAX-1 defizienten Zellen zu einem Anstieg des Bif-1 Levels und dessen phosphorylierter Form führte. Zusammenfassend kann festgestellt werden, dass die vorliegende Studie erstmalig die gemeinsame Interaktion von Hax-1 und Bif-1 belegt und diese, die durch Autophagie hervorgerufenen Zelltod, nach *EBSS-Starvation* reguliert.

HAX1: **Hematopoietic Cell Specific Lineage substrate 1 associated protein X 1**

Bif-1: **BAX interacting Factor 1**

EBSS: **Earle’s Balanced Salt solution**

Synopsis

The 'Kostmann gene' encodes a protein designated as HAX-1, for HS-1-associated protein X, a mitochondria targeted protein (35KDa intracellular protein).

Bif-1 member of the SH3 domain family of endophilins and Serpin b1a member of the Ova- Serpin superfamily were fished out by the Yeast Tow hybrid screen using HAX1 as bait.

Over expression of HAX1 in E.coli was not possible probably because of the presence of rare codons and codon bias. HAX1 was found to be unstable when overexpressed and purified in Dictyostelium discoideum.

Bif-1 and HAX1 were found to interact with each other endogenously in 293T and Jurkat cell lines The C-terminal 179 to 234 amino acids of HAX1 were proved to be involved in binding to Bif-1. Significant amounts of endogenous HAX1 and Bif-1 were found to Co-localize in the cytoplasm. Loss of HAX1 leads to autophagic cell death upon EBSS starvation in HAX1 deficient patient fibroblasts. Loss of HAX1 leads to an increase in the levels of Bif-1 and its phosphorylated form upon EBSS starvation. In conclusion, the present study for the first time reports HAX1 interacts with Bif-1 and regulates autophagic cell death upon EBSS starvation

HAX1: **Hematopoietic Cell Specific Lineage substrate 1 associated protein X 1**

Bif-1: **BAX interacting Factor 1**

EBSS: **Earle's Balanced Salt solution**

Abbreviations

°C	**Degree Celsius**
µg	**Microgram**
mg	**Milligram**
ml	**Milliliter**
mM	**Millimolar**
M	**Molar**
L	**Liter**
µl	**Microliter**
sec	**Second**
µM	**Micromolar**
hr	**Hour**
min	**Minute**
mA	**Milliampere**
V	**Volt**
RT	**Room temperature**
Rpm	**Rotation per minute**
APS	**Ammonium peroxodisulfate**
TEMED	**N, N, N,-Tetramethylethylenediamine**
EDTA	**Ethylendiamine tetraacetate**
EGTA	**Ethylenglycol tetraacetate**
Tris	**Tris (hydroxymethyl)-aminoethane**
IPTG	**Isopropyl β–D-1-thiogalactopyranoside**
DTT	**Dithiothreitol**
DMSO	**Dimethyl sulphoxide**
DMF	**Dimethyl Formamide**
PMSF	**Phenylmethylsulphonyl fluoride**
FPLC	**Fast performance liquid chromatography**
GFP	**Green fluorescent protein**
HRP	**Horse radish peroxidase**

LB	**Luria broth**
TBST	**Tris buffer saline Tween-20**
PBS	**Phosphate buffer saline**
TAE	**Tris acetate EDTA**
SDS	**Sodium dodecyl sulfate**

Yeast abbreviation

AD/library	**a fusion of GAL4 AD with library cDNA/ protein DNA-**
BD/ bait	**A fusion of the GAL4-BD with bait protein**
Yeast phenotypes	
Ade^-, His^-, Leu^-, or Trp^-	**requires adenine, histidine, leucine or tryptophan in the medium to grow; is auxotrophic for at least one of these specific nutrients**
Miscellaneous	
Ade2p	**Protein encoded by the yeast ADE2 gene**
3-AT	**3-amino-1, 2, 4-triazole; a competitive inhibitor of the His3 protein**
DO	**Dropout (supplement or solution); a mixture of specific amino acids and nucleosides used to supplement SD base to make SD medium; DO solutions are missing one or more of the nutrients required by untransformed yeast to grow on SD medium.**
His3p	**protein encoded by the yeast His3 gene**
SD medium	**Minimal Synthetic Dropout medium; comprised of a nitrogen base, a carbon source (glucose or galactose) and a DO supplement.**
Bif-1	**BAX interacting factor 1**
UVRAG	**Ultraviolet Irradiation resistance-associated gene**
PI(3)KC(3)	**Class III Phosphatidylinositol 3-Kinase**

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1. INTRODUCTION

1.1 Cancer

Cancer Research has generated a rich and complex body of knowledge, revealing cancer to be a disease involving dynamic changes in the genome. The foundation has been set in the discovery of mutations that produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function; both classes of cancer genes have been identified through their alteration in human and animal cancer cells and their elicitation of cancer phenotypes in experimental models(Bishop 1996)

Research over the past decades has revealed a small number of molecular, biochemical, and cellular traits-acquired capabilities-shared by most and perhaps all types of human cancer. Our faith in such simplification derives directly from the teachings of cell biology that virtually all mammalian cells carry a similar molecular machinery regulating their proliferation, differentiation, and death(Hanahan and Weinberg 2000)

The barriers to development of cancer are embodied in teleology: cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis (Hanahan and Weinberg 2000). There are more than 100 distinct types of cancer, and subtypes of tumors can be found within specific organs (Hanahan and Weinberg 2000). This complexity provokes a number of questions. How many distinct regulatory circuits within each type of target cell must be disrupted in order for such a cell to become cancerous? Does the same set of cellular regulatory circuits suffer disruption in the cells of the disparate neoplasms arising in the human body? Which of these circuits operate on a cell-autonomous basis, and which are coupled to the signals that cells receive from their surrounding microenvironment within a tissue? Can the large and diverse collection of cancer associated genes be tied to the operations of a small group of regulatory circuits? (Hanahan and Weinberg 2000)

1.2 Cell death

An Intact cell death pathway is required for successful embryonic development and the maintenance of normal tissue homeostasis. Cell death has proven to be tightly interwoven with other essential cell pathways. The identification of critical control points in the cell

death pathway has yielded fundamental insights for basic biology, as well as provided rational targets for new therapeutics (Danial and Korsmeyer 2004)

Cell death can be physiologic or pathologic. Three major types of cell death have been reported in mammalian cells. They are Apoptosis (Type1 programmed cell death)(Kerr, Wyllie et al. 1972; Kroemer, El-Deiry et al. 2005; Galluzzi, Maiuri et al. 2007), Autophagy (Type2 Programmed cell death)(Klionsky 2007) and Necrosis (Kerr 1971; Majno and Joris 1995). Although many new subtypes have been reported, most of them would fall into one of the above three mentioned categories.

1.3 TYPE 1 PROGRAMMED CELL DEATH (APOPTOSIS)

Physiologic cell death in animal species generally occurs through a mechanism commonly called "apoptosis," typically involving activation of intracellular proteases known as caspases.(Kerr, Wyllie et al. 1972) Apoptosis is a highly conserved mechanism that has evolved to maintain cell numbers and cellular positioning within tissues comprised of different cell compartments. Programmed cell death (apoptosis) was first described in 1972 by Currie and colleagues. (Kerr, Wyllie et al. 1972) It is a common type of cell death associated with morphological features that had been repeatedly observed in various tissues and cell types. Apoptosis is an essential part of life for multicellular organisms that plays an important role in development and tissue homeostasis (Prindull 1995). During development many cells are produced in excess which eventually undergo programmed cell death and thereby contribute to sculpturing organs and tissues.(Meier, Finch et al. 2000) Apoptosis is delicately regulated and balanced in a physiological context. Failure of this regulation results in pathological conditions such as developmental defects, autoimmune diseases, neurodegeneration or cancer.(Thompson 1995) Characteristic apoptotic features include cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation, finally ending with the engulfment by macrophages or neighboring cells, thereby avoiding an inflammatory response in surrounding tissues.(Savill and Fadok 2000) Apoptosis is distinct from necrosis in which the cells suffer a major insult, leading to a loss of membrane integrity, swelling and disruption of the cells.

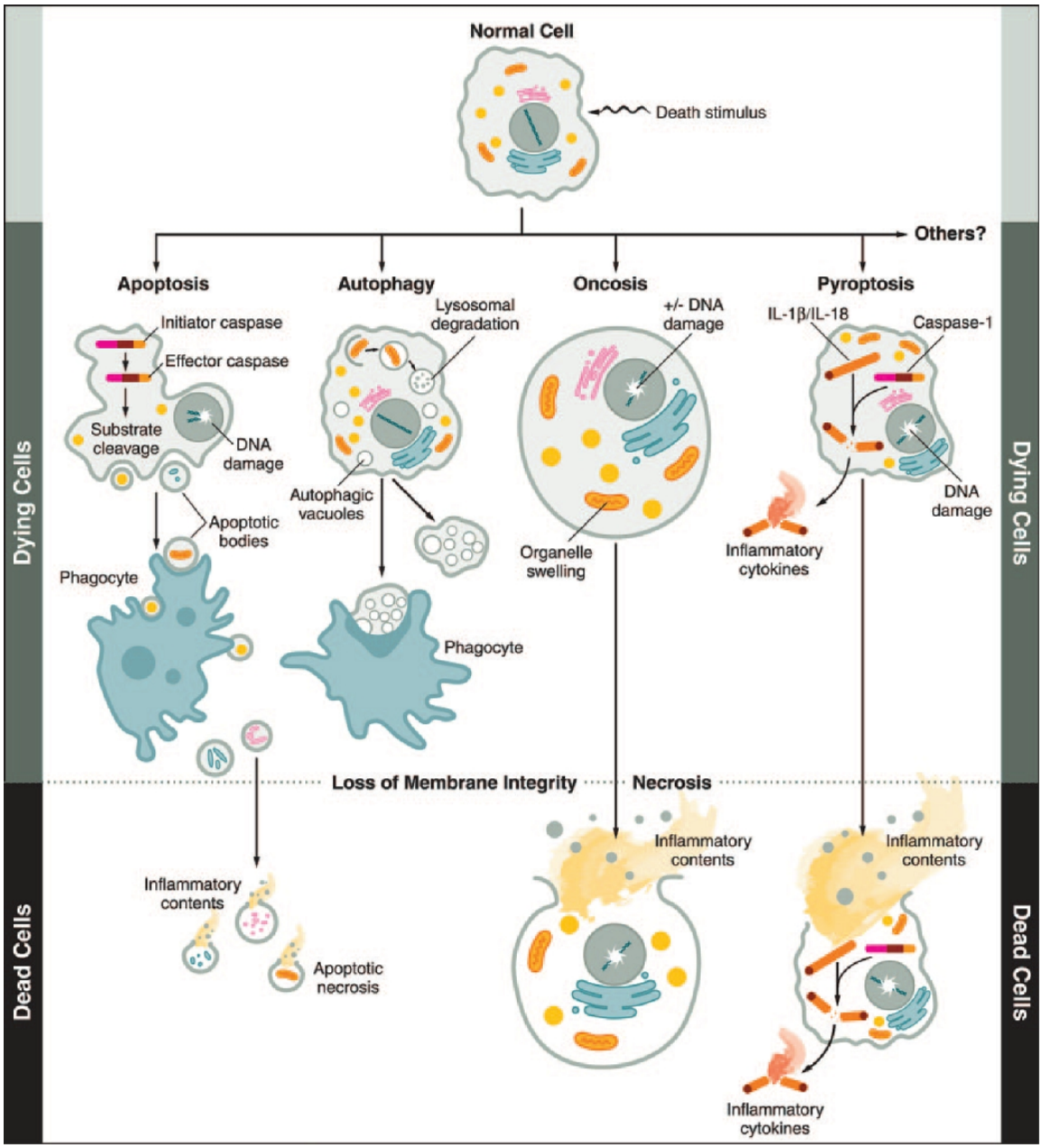


Figure 1: Pathways leading to cell death: Healthy cells respond to death-inducing stimuli by initiating a variety of molecular pathways leading to cell death. Completion of the proper pathway is a critical cellular function to ensure that the appropriate outcome is ultimately achieved in a multicellular organism. Failure to die in response to particular stimuli can result in abortive embryogenesis and organ dysfunction and contributes to the initiation of cancer. Proinflammatory death is vital in triggering appropriate immune responses or, in the extreme, may cause tissue pathology and organ dysfunction. Therefore, pathway utilization can dramatically influence biological systems. Apoptosis is a pathway leading to cell death that features the activation of initiator caspases that activate effector caspases to cleave cellular substrates. Apoptotic cells demonstrate cytoplasmic and nuclear condensation, DNA damage, formation of apoptotic bodies, maintenance of an intact plasma membrane, and exposure of surface molecules targeting intact cell corpses for phagocytosis. In the absence of phagocytosis, apoptotic bodies may proceed to lysis and secondary or apoptotic necrosis. Autophagy features degradation of cellular components within the intact dying cell in autophagic vacuoles. The morphological characteristics of autophagy include vacuolization, degradation of cytoplasmic contents, and slight chromatin condensation. Autophagic cells can also be taken up by phagocytosis. Oncosis is the prelethal pathway leading to cell death accompanied by cellular and organelle swelling and membrane breakdown, with the eventual release of inflammatory cellular contents. Pyroptosis is a pathway to cell death mediated by the activation of caspase-1, a protease that also activates the inflammatory cytokines, IL-1_, and IL-18. This pathway is therefore inherently proinflammatory. Pyroptosis also features cell lysis and release of inflammatory cellular contents (Cookson 2005).

During necrosis, the cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue (Leist and Jaattela 2001).

Intensive effort has been made to explore the molecular mechanisms of the apoptotic signaling pathways including the initiation, mediation, execution, and regulation of apoptosis (Jin and El-Deiry 2005). The original understanding of cell death has come from genetic studies in the nematode Caenorhabditis *elegans*, which as a model system contains the basic components of the cell death machinery. Activation and regulation of apoptosis in higher organisms depends on corresponding analogous components found in C. *elegans* but with more complexity.

In mammals, a wide array of external signals may trigger two major apoptotic pathways, namely the extrinsic pathway (death receptor pathway) or the intrinsic pathway (the mitochondrial pathway) within a cell (Green, Knight et al. 2004). The extrinsic pathway is activated by apoptotic stimuli comprising extrinsic signals such as the binding of death inducing ligands to cell surface receptors. In other cases, apoptosis is initiated following intrinsic signals including DNA damage induced by irradiation or chemicals, growth factor deprivation or oxidative stress. In general intrinsic signals initiate apoptosis via the involvement of the mitochondria (Green, Knight et al. 2004).

1.4 Extrinsic and Intrinsic pathways

The extrinsic pathway is mediated by cell surface death receptors, such as Fas, tumor necrosis factor receptor, or Tumor necrosis (TNF)-related apoptosis-inducing ligand (TRAIL) receptors (Ashkenazi and Dixit 1998). Death ligand stimulation results in oligomerization of the receptors and recruitment of the adaptor protein Fas-associated death domain (FADD) and caspase-8, forming a death-inducing signaling complex (DISC) (Ashkenazi and Dixit 1998). Autoactivation of caspase-8 at the DISC is followed by activation of effector caspases, including caspase-3, -6 and -7, which function as downstream effectors of the cell death program (Ashkenazi and Dixit 1998). The intrinsic pathway is mediated by diverse apoptotic stimuli, which converge at the mitochondria. Release of cytochrome c from the mitochondria to the cytoplasm initiates a caspase cascade. Cytosolic cytochrome c binds to apoptosis protease-activating factor 1 (Apaf-1) and procaspase-9, generating an intracellular DISC-like complex known as

"apoptosome". Within the apoptosome, caspase-9 is activated, leading to processing of caspase-3(Shi 2002). The two pathways of apoptosis, extrinsic/death receptor and intrinsic/mitochondrial, converge on caspase-3 and subsequently on other proteases and nucleases that drive the terminal events of programmed cell death (Jin and El-Deiry 2005). The last steps of apoptosis include packaging of cell content into apoptotic bodies and phagocytosis. Phosphatidylserine is normally present on the cytoplasmic side of the cell membrane, but exposure to the external surface of the cell membrane occurs when a cell undergoes apoptosis (Savill and Fadok 2000). This event triggers the phagocytosis reaction (Savill and Fadok 2000). Cellular apoptosis is tightly controlled by a complex regulatory network. Pro-survival signals enhance the expression and/or activity of anti-apoptotic regulatory molecules thereby keeping in check the activation of pro-apoptotic factors. A set of various anti-apoptotic molecules and mechanisms has been identified, such as NF-kB, AKT, Bcl-2 and the IAP family of proteins. Every step in the apoptotic cascade is monitored and controlled by certain pro-survival signals (Vogelstein and Kinzler 2004). Pro-apoptotic factors can counteract those inhibitory molecules when apoptotic demise of a cell is timely and imperative.

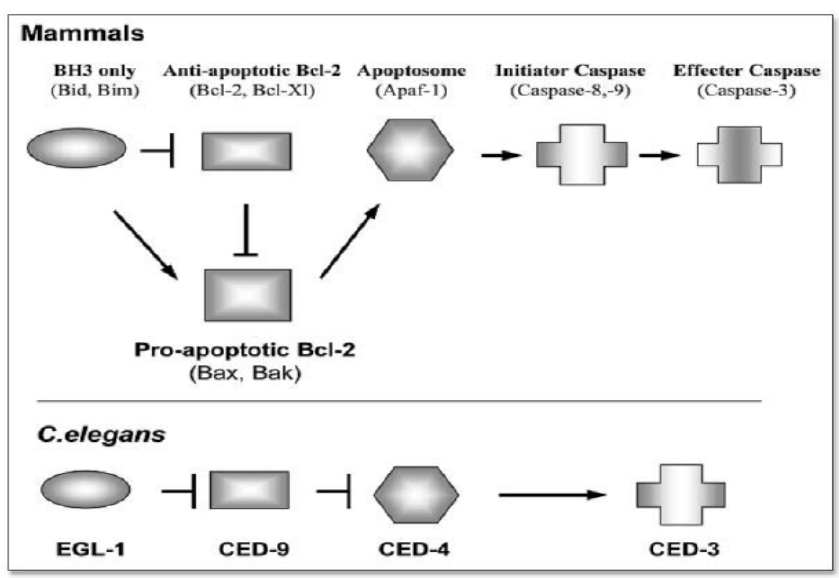


Figure 2: Comparison of mammals to the *C. Elegans* system. Several genes have been identified that function in apoptotic killing in *C. elegans*. These cell deaths rely on the presence of CED-3 (caspase homologue), and CED-4 (Apaf-1 homologue) that binds to and activates CED-3. In healthy cells, CED-4 remains inactive by its association with CED-9 (antiapoptotic Bcl-2 homologue). The protein EGL-1 (BH3 only member homologue) is a trigger of cell death and is expressed in response to certain developmental cues. EGL-1 binds to CED-9, displacing CED-4, which in turn activates CED-3 to induce apoptosis. *C. elegans* as a model system contains

the basic components of the cell death machinery. Activation and regulation of apoptosis in higher organisms depends on corresponding analogous components found in *C.elegans* but with more complexity (Jin and El-Deiry 2005)

1.5 TYPE II PROGRAMMED CELL DEATH (AUTOPHAGY)

Autophagy is generally defined as a lysosome-dependent mechanism of intracellular degradation that is used for the turnover of cytoplasm (Xie and Klionsky 2007). Several forms of autophagy have been described, including macroautophagy(Klionsky 2005; Yorimitsu and Klionsky 2005), microautophagy(Kunz, Schwarz et al. 2004), chaperone-mediated autophagy (Majeski and Dice 2004), and piecemeal microautophagy of the nucleus (Kvam and Goldfarb 2007). Macroautophagy is used for the sequestration and degradation of cytoplasm in a process that uses specialized cytosolic vesicles that ultimately fuse with the lysosome. By contrast, microautophagy involves direct uptake of cytoplasm at the lysosome surface by invagination of the limiting membrane of the lysosome. Chaperone-mediated autophagy also takes place at the lysosome membrane, but relies on translocation of unfolded proteins across the membrane. Piecemeal microautophagy of the nucleus is a selective type of autophagy that occurs in yeast that is used to degrade portions of the nucleus by taking them into vacuole (the yeast analogue of the lysosome) lumen (Xie and Klionsky 2007).

In mammals, autophagy has been observed in many tissues, and has been shown to be essential for differentiation and development as well as for cellular maintenance (Arico, Petiot et al. 2001). In addition, autophagy has been shown to have significant associations with neurodegenerative diseases, cardiomyopathies, cancer, programmed cell death, and bacterial and viral infections (Dorn, Dunn et al. 2002; Baehrecke 2003; Cuervo 2003; Edinger and Thompson 2003). During the autophagic process, a cup-shaped structure also referred to as an isolation membrane or preautophagosome forms in the cytosol. It engulfs cytosolic components, including organelles, and later become enclosed to form an autophagosome. The autophagosome subsequently fuses with a lysosome, enabling the intra-autophagosomal components to become degraded by lysosomal hydrolytic enzymes (Tanida, Ueno et al. 2004).

Autophagy occurs at a basal rate in most cells, where it acts as a cytoplasmic quality control mechanism to eliminate protein aggregates and damaged organelles (Mizushima 2005). The physiological importance of basal autophagy in maintaining tissue homeostasis has been recently demonstrated in conditional brain and liver ATG knockout

mouse models (Komatsu, Waguri et al. 2005; Hara, Nakamura et al. 2006; Komatsu, Waguri et al. 2006). The proteins involved in the autophagic process in yeast have been isolated and characterized (Tsukada and Ohsumi 1993; Thumm, Egner et al. 1994; Harding, Morano et al. 1995). Recently nomenclature for Yeast **A**u**T**opha**G**y-related genes has been unified to ***ATG*** from ***APG***, ***AUT,*** *and* ***CVT;*** Many mammalian homologues of yeast *Atg* genes have been identified and characterized, suggesting that the molecular mechanisms of autophagy have been conserved from yeasts to mammals(Klionsky, Cregg et al. 2003). Many mammalian homologues of yeast *Atg* genes have been identified and characterized, suggesting that the molecular mechanisms of autophagy have been conserved from yeasts to mammals (Tanida, Ueno et al. 2004).

Autophagy begins with a pre-autophagosomal structure of unknown origin that gives rise to an ‘‘isolation’’ membrane or phagophore (M. Fengsrud 2004; Suzuki and Ohsumi 2007). This membrane elongates to form the autophagosome, which is a double membrane-bound structure in the 0.5-1.5 μm range in mammalian cells. Once this has been formed, the autophagosomes can receive input from the endocytic pathway to form a hybrid organelle known as the amphisomes (Stromhaug and Seglen 1993; M. Fengsrud 2004).

Although autophagy is recognized as being a cytoprotective process, for some years it has also been suspected of being involved in type-2 Programmed Cell Death (PCD) or autophagic cell death (as distinct from type-1 PCD or apoptosis)(Clarke 1990). Only recently, genetic studies have shown that cells can be killed by autophagy when apoptosis is inhibited (Shimizu, Kanaseki et al. 2004; Yu, Alva et al. 2004; Levine and Yuan 2005). Moreover, in a different context it has been shown that autophagy contributes with apoptotic signals to the killing of cells (Baehrecke 2003; Crighton, Wilkinson et al. 2006; Djavaheri-Mergny, Amelotti et al. 2006; Espert, Denizot et al. 2006; Gozuacik and Kimchi 2007) and elimination of apoptotic cells by phagocytic cells (Qu, Zou et al. 2007).

Recent studies suggest that the regulation of autophagy overlaps with that of apoptosis, and that signaling molecules may modulate several different players in the autophagic molecular machinery (Codogno and Meijer 2005; Gozuacik and Kimchi 2007).

Atg proteins involved in the formation of autophagosomes are almost entirely conserved in eukaryotic cells (Klionsky, Cregg et al. 2003). These proteins can be divided into four

functional groups: (1) the Atg1 complex that integrates signaling from the TOR (Target of Rapamycin) kinase. This complex is involved in several steps during the initiation, nucleation and expansion steps of autophagosome formation. (2) The Beclin 1 complex (the ortholog of the yeast protein Atg6)/class III PI3K (hVps34) is involved in the nucleation phase. (3) Two ubiquitin-like conjugation systems, the Atg12 and the LC3 systems (LC3 the ortholog of the yeast protein Atg8) act sequentially during autophagosome formation (Ohsumi 2001). Atg12 conjugates Atg5 during the initiation step, and promotes the conjugation of LC3 to phosphatidylethanolamine that is required during the elongation step. (4) A recycling pathway controlled by Atg1 and Atg9 that mediates the shuttling of Atg proteins in and out of the autophagosomal membranes during the formation of autophagosomes(Reggiori, Shintani et al. 2005; Young, Chan et al. 2006). The maturation of autophagosomes requires the activity of Rab GTPases (Rab 7 and Rab 24) (Munafo and Colombo 2002; Gutierrez, Munafo et al. 2004; Jager, Bucci et al. 2004), and of other factors that are involved in fusion events during vesicular trafficking in the endocytic pathway (Nara, Mizushima et al. 2002; Atlashkin, Kreykenbohm et al. 2003; Esselens, Oorschot et al. 2004).

1.5.1 mTOR complexes

Target of Rapamycin (TOR) is a conserved Ser/Thr protein kinase that regulates cell growth, cell cycle progression, nutrient import, protein synthesis and autophagy (Sarbassov, Ali et al. 2005; Arsham and Neufeld 2006; Wullschleger, Loewith et al. 2006; Bhaskar and Hay 2007; Lee, Inoki et al. 2007). Rapamycin binds to the FKBP12 protein to form a complex that interacts and inhibits several functions regulated by TOR (Wullschleger, Loewith et al. 2006). Apart from yeast, where two TOR genes, TOR1 and TOR2, have been identified, all eukaryotic genomes examined so far contain a single TOR gene. Four functional domains are conserved in TOR proteins including the central FAT (FRAP, ATM, TTRAP) domain, the FRB (FKBP12-rapamycin binding domain) domain, the kinase domain and at the most C-terminal part of the protein the FATC domain (Sarbassov, Ali et al. 2005; Wullschleger, Loewith et al. 2006; Bhaskar and Hay 2007).

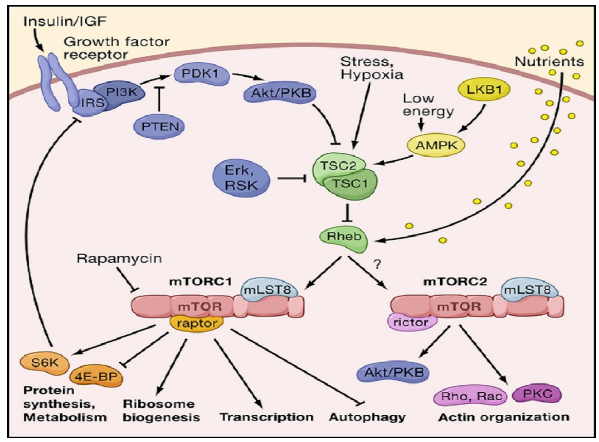


Figure 3: Model of the mTOR Signaling Network in Mammalian Cells: The mTOR signaling network consists of two major branches, each mediated by a specific mTOR complex (mTORC). Rapamycin-sensitive mTORC1 controls several pathways that collectively determine the mass (size) of the cell. Rapamycin- insensitive mTORC2 controls the actin cytoskeleton and thereby determines the shape of the cell. MTORC1 and possibly mTORC2 respond to growth factors (insulin/IGF), energy status of the cell, nutrients (amino acids), and stress. mTORC1 (and likely mTORC2) are multimeric, although are drawn as monomers. Arrows represent activation, whereas bars represent inhibition (Wullschleger, Loewith et al. 2006)

1.5.2 Regulation of Autophagy by TOR

Autophagy begins when the cell perceives a reduction in the availability of nutrients. The molecular mechanisms by which this starvation signal is transmitted to the autophagic machinery have been elucidated from studies performed in yeast. TOR has been identified as an essential component in the regulation of autophagy. Inactivation of TOR function by rapamycin induces autophagy even in rich nutrient conditions, indicating that TORC1 inhibits autophagy (Noda and Ohsumi 1998). TORC1 inactivation induces autophagy primarily at the level of autophagosome formation by negatively regulating the association between the Ser/Thr protein kinase ATG1 with ATG13, a regulatory subunit of ATG1 (Kamada, Funakoshi et al. 2000), (Fig. 8). The ATG1 kinase plays a pivotal role in the control of autophagy and its activity is required for the switch from the formation of Cvt (cytoplasm-to-vacuole targeting) vesicles to the formation of autophagosomes (Scott, Hefner-Gravink et al. 1996; Matsuura, Tsukada et al. 1997).

Several autophagy-related proteins have tumor suppressor activity (Beclin 1, Atg5, Bif-1, Atg4C, UVRAG), and some autophagy gene mutations, detected in humans or induced in cellular or animal models, can lead to an accumulation of DNA damage and genome instability (Mathew, Kongara et al. 2007). In cancer progression, autophagy may, on the other hand, provide tumor cells with a survival strategy, thus suggesting a therapeutic use

for autophagy down regulation in solid tumors (Levine, Sinha et al. 2008; Mizushima, Levine et al. 2008).

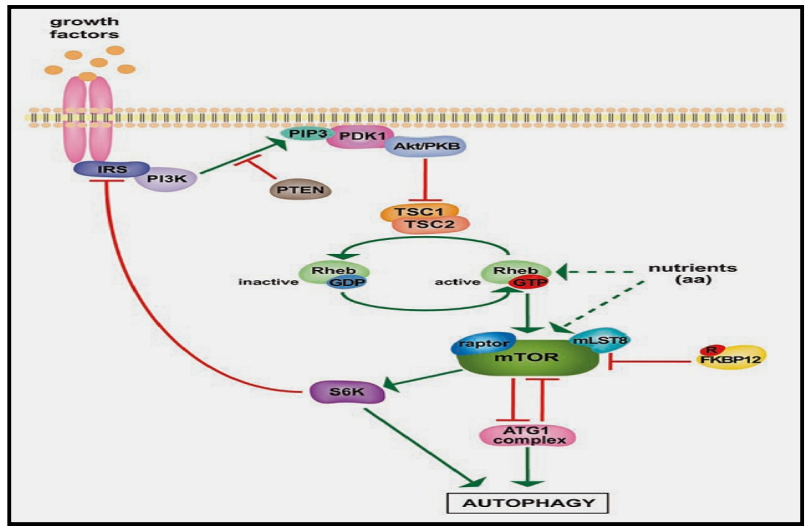


Figure 4: Control of autophagy by the TOR signaling network: mTORC1 receives inputs from intracellular and extracellular stimuli, including nutrients (amino acids) and growth factors (insulin). Amino acids may activate the GTPase Rheb which positively regulates mTORC1 while the growth factor signal is transduced to the tumor suppressor complex TSC1/TSC2 via the insulin signaling pathway. TSC1/TSC2 negatively regulates mTORC1 by inactivating Rheb. Once activated, mTORC1 inhibits autophagy by acting on the ATG1 complex. Studies performed in Drosophila indicate that ATG1 negatively feeds back on TOR signaling by an unknown mechanism. S6K may contribute to the basal activity of autophagy via its feedback inhibition of the PI3K-dependent signaling pathway. Green arrows represent activation, whereas red bars represent inhibition. Dashed lines refer to potential interactions. (Sandra Díaz-Troya 2008).

In theory, there could be a link between apoptosis and autophagy in these cases, suggesting that autophagy may trigger cell demise upon strong environmental or extrinsic stress during development, thereby matching the scenario believed to occur in lower eukaryotes. However, the mechanisms and functions of autophagy need further investigation. The question remains as to whether autophagy constitutes an understudied form of cell destruction or, alternatively or in parallel, whether it is a regulated process capable of rapidly changing the cell in response to environmental stimuli or intrinsic metabolic needs (Cecconi and Levine 2008).

The molecular mechanisms of apoptosis and autophagy are quite different and involve fundamentally distinct sets of regulatory and executioner molecules (Danial and Korsmeyer 2004; Levine and Klionsky 2004; Mizushima and Klionsky 2007). Thus far, there is no known overlap between the proteins that are essential for autophagy in *Saccharomyces cerevisiae* and those that regulate programmed cell death in this species (Buttner, Eisenberg et al. 2006). However, in mammals (and likely other metazoans),

there is one family of proteins, the so-called Bcl-2 protein family, that plays a dual role in the control of apoptosis and autophagy. Although Bcl-2 family proteins were initially characterized as cell death regulators, it has recently become clear that they also control autophagy (Levine, Sinha et al. 2008).

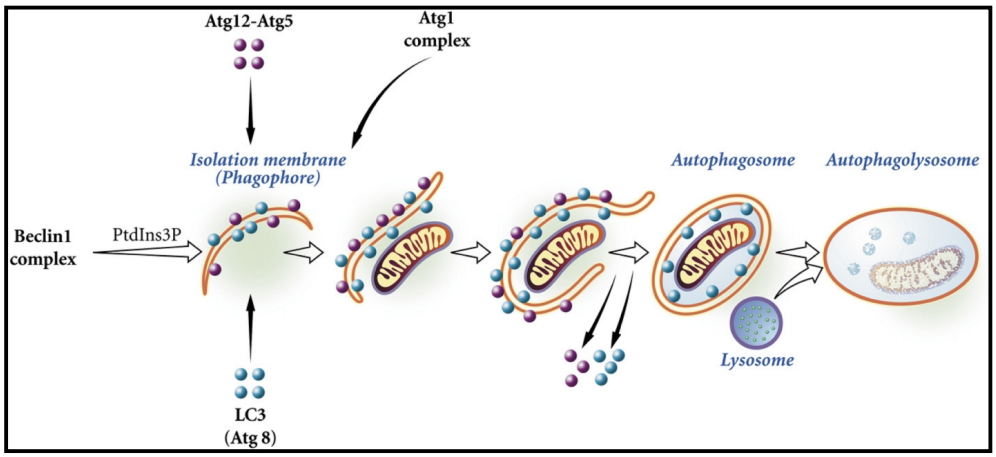


Fig 5: Initiation of Autophagosome due to the production of PtdIns3P by Beclin complex: The production of PtIns3P by the Beclin 1/hVps34 complex is an early event in autophagosome formation. This first step is essential for the recruitment of other Atg proteins at the isolation membrane or phagophore. Thereafter, sequential recruitment of Atg12,Atg5 and LC3 (Atg8) conjugates occurs at the isolation membrane. The LC3-I form is covalently bound to phosphatidylethanolamine (PE) a lipid in the autophagosomal membrane to form LC3-II. After completion, most of the Atg proteins, except a fraction of LC3-II bound to the luminal side of the autophagosomal membrane are recycled to the cytosol. The Atg1 complex may be implicated in different steps of the formation of autophagosomes to regulate the transport of Atg proteins to and from the isolation membrane. The last step in this process is the fusion between autophagosomes and lysosomes, and the degradation of the sequestered material, including LC3-II (Sophie Pattingre 2007).

1.5.3 Role of Beclin1 in Autophagy

Beclin 1 (the mammalian ortholog of yeast Atg6) was originally discovered as a Bcl-2-interacting protein (Liang, Kleeman et al. 1998) and was the first human protein shown to be indispensable for autophagy (Liang, Jackson et al. 1999). The overall structure of Atg6/Beclin 1, as well as its essential role in autophagosome formation, is evolutionarily conserved throughout all eukaryotic phyla (Levine, Sinha et al. 2008). In mammals, the interaction of Beclin 1 with Vps34 and Vps15 is conserved (Kihara, Kabeya et al. 2001). Additional Beclin 1 interactors include UVRAG, Ambra1 and Bif-1 (also called endophilin B1) (Liang, Feng et al. 2006; Fimia, Stoykova et al. 2007; Takahashi, Coppola et al. 2007). Knockdown and knockout studies performed on human and mouse cells indicate that UVRAG, Ambra1 and Bif-1 are all indispensable for the activation of

autophagy, as well as for the optimal activation of Vps34. UVRAG and Ambra1 directly interact with distinct regions of the Beclin 1 molecule (Liang, Feng et al. 2006; Fimia, Stoykova et al. 2007), While Bif-1 interacts with Beclin 1 indirectly via UVRAG (Takahashi, Coppola et al. 2007).

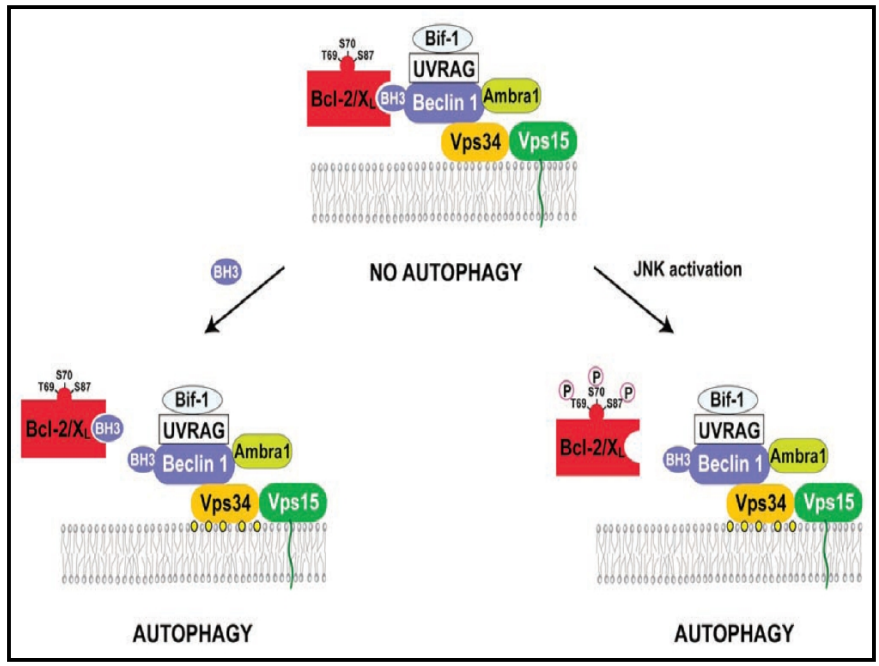


Figure 6.

The Beclin 1 interactome in human cells and possible mechanisms underlying the regulation of its autophagy function.

Beclin 1 binds to the Class III phosphatidylinositol kinase, Vps34, which is associated with the myristoylated, membrane-anchored kinase, Vps15. The lipid kinase activity of the multiprotein Beclin 1/Vps34 complex converts phosphatidylinositol into phosphatidylinositol-3-phosphate (depicted as yellow circles), which is involved in the nucleation of pre- autophagosomal structures. Beclin 1 interacts with several coactivators including UVRAG, Ambra1 and Bif-1, as well as with the inhibitors, Bcl-2 and Bcl-XL. Shown here are two mechanisms for disrupting the interaction between Beclin 1 and Bcl-2 or Bcl-XL, and thereby activating autophagy: (1) Proteins that contain BH3 domains or small molecules that mimic BH3 domains can bind to the BH3-binding groove of Bcl-2 or Bcl-XL and competitively disrupt the interaction between Bcl-2 or Bcl-XL and Beclin 1. This leads to the de-inhibition of the lipid kinase activity of the class III phosphatidylinositol-3- kinase Vps34 and autophagy induction. (2) C-Jun-N'-terminal kinase 1 (JNK1)-mediated phosphorylation of Bcl-2 during starvation can disrupt its interaction with Beclin 1, leading to autophagy induction (Levine, Sinha et al. 2008).

However, the relative contribution of these molecules to autophagy (and perhaps autophagy-unrelated functions) is not equivalent. The deletion of only one *beclin 1* allele, which causes a reduction of Beclin1 protein expression by 50%, is sufficient to cause a severe defect in autophagy (Qu, Yu et al. 2003; Yue, Jin et al. 2003), a phenotype that is only observed when both alleles of *Ambra1* or *bif-1* are deleted (Liang, Feng et al. 2006; Fimia, Stoykova et al. 2007). *Beclin 1*-/- mice succumb early during embryonic development (around day e7.5), (Qu, Yu et al. 2003; Yue, Jin et al. 2003), while *ambra1*-

/- mice die from exencephaly during birth,(Fimia, Stoykova et al. 2007) and *bif-1*-/- mice are viable(Takahashi, Coppola et al. 2007). A common denominator of the knockout phenotypes is that *beclin1*+/- as well as *bif-1*-/- mice tend to develop spontaneous tumors (Qu, Yu et al. 2003; Yue, Jin et al. 2003; Takahashi, Coppola et al. 2007), suggesting that a functional Beclin 1 complex (and by extension autophagy) may participate in tumor suppression.

1.6 HAX1

HAX1 is named "HS1 interacting protein 1" because HAX1 was first discovered via a yeast two-hybrid screen with HS1, which is a member of the Src family of kinases and plays roles in proliferation and apoptosis of lymphoid cells (Suzuki, Demoliere et al. 1997). The 'X' comes from the "X-Gal colony filter assay", used in the yeast two-hybrid experiment (Schaffer and Klein 2007). HAX1 is more recently named as "Kostmann Gene" after it was reported that Loss of HAX1 leads to Kostmann like Severe Congenital Neutropenia (Klein, Grudzien et al. 2007)

The human HAX1 gene is located on chromosome 1 (1q21) within the epidermal differentiation complex. Pseudogenes are present in the mouse and rat genomes along with a processed pseudogene on human chromosome X. Moreover, sequences similar to HAX1 were found in numerous other species, including zebra fish (*Danio rerio*) but not in more primitive organisms such as the nematode, Caenorhabditis elegans (Chao, Parganas et al. 2008) It has been reported that cytokines induce HAX1 expression in murine lymphocytes; Interleukin (IL)-2 induced the expression of HAX1 in pre-activated T cells from the spleen, and in pro-B cells derived from the bone marrow IL-7 treatment triggered an upregulation of HAX1 expression.

To date, alternative splicing of the HAX1 gene has been analyzed in rat (Grzybowska, Sarnowska et al. 2006), mouse(Cho, Adamson et al. 2002; Lees, Hart et al. 2008), and human(Carlsson, van't Hooft et al. 2008; Lees, Hart et al. 2008) genomes. Human and Rat splice variants bear some similarity; however, the mouse variants identified so far appear to be distinct, although it seems probable that the extended analysis of the mouse transcriptome may reveal rat or human counterparts. In rat and humans there are two main sources of diversity of the splice variants: the use of the internal splice sites in exon

2 and intron retention (mainly intron 1). The internal splice sites generate variants without various parts of exon 2. In some patients with Kostmann syndrome were indentified to have mutations in the region encompassing the part of HAX1 removed by alternate splicing in human variant II. A different internal splice site in exon 2 is used in human splice variant VI, introducing a frameshift and resulting in a different open reading frame at the C-terminal part of the putative protein. There are two variants in rat generated through the internal splice site within exon 2, which resulted in the removal of various parts of exon 2 (variant II and VIII). Another rat variant (IX) does not contain exon 2 at all. In mice with Burn injury a truncated HAX1 transcript (HAX1xs) was expressed rapidly and was found to be transiently induced in multiple tissues (Cho, Adamson et al. 2002), but the deletion in this transcript removes part of the exon 2 and the whole of exon 3, and therefore does not represent an exact counterpart of either rat or human variants(Fadeel and Grzybowska 2009).

Retention of intron 1 was observed in one human variant and five rat variants. (Grzybowska, Sarnowska et al. 2006; Carlsson, van't Hooft et al. 2008; Lees, Hart et al. 2008) In the three rat variants (V, VI and VII) and one human variant (III) retention of intron 1 causes changes in the 5′ sequence, shifting downstream the start codon of the main open reading frame and creating several short, upstream open reading frames (uORFs) preceding the main reading frame. Alternative splicing occurs mostly in the 5′ end of the HAX1 transcript, thus modifying the N-terminal part of the protein; this generates truncated isoforms devoid of the putative mitochondrial targeting sequence (Grzybowska, Sarnowska et al. 2006) or the entire C-terminal part of the protein, isoforms without acid box and Bcl-2 homology (BH) domains, or with alterations in the PEST (proline, glutamic acid, serine, threonine) domain (Fig 7). The functional significance of the putative isoforms remains an open question, although there are recent indications of isoform-dependent neurodevelopmental abnormalities in patients with Kostmann disease (Carlsson, van't Hooft et al. 2008; Germeshausen, Grudzien et al. 2008) However, information is lacking on the expression of different isoforms at the protein level(Fadeel and Grzybowska 2009).

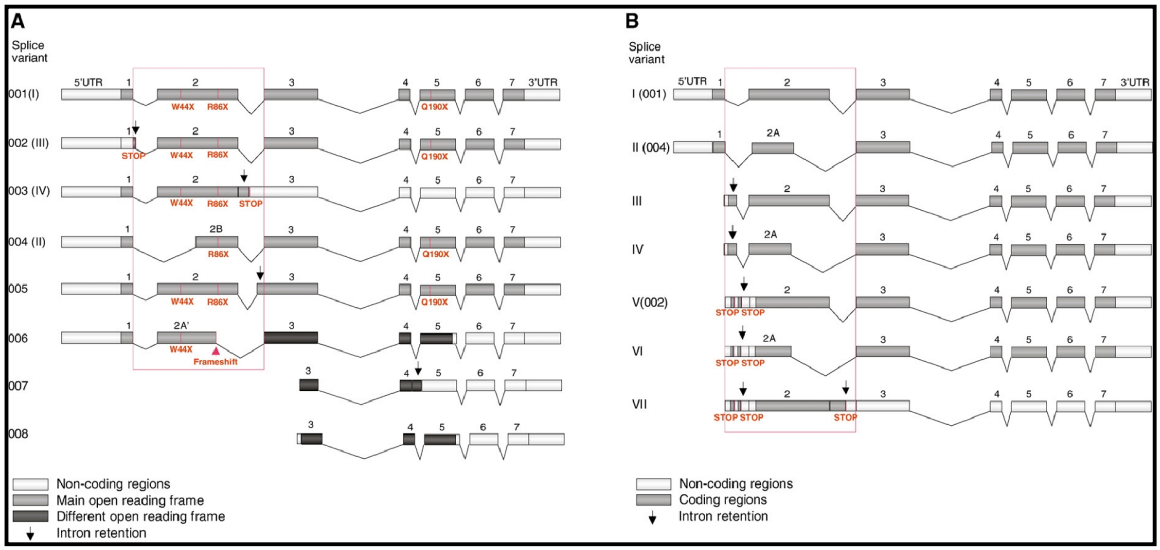


Fig 7: Splice variants of A) Human and B) Rat HAX1: (A) Alternative splicing of the human HAX1 gene occurs mostly in the region containing intron 1 – exon 2 – intron 2 (red box). The only functional significance that has been assigned thus far to this pattern of splicing emerges from the fact that the HAX1 mutation, W44X (introducing a stop codon, vertical red line) identified in SCN patients without CNS symptoms is spliced out in transcript variant II (Klein, Grudzien et al. 2007). The R86X mutation that has been identified in SCN patients with CNS symptoms (Carlsson and Fasth 2001) is also shown (introducing a stop codon, vertical red line). The "classical" Swedish Kostmann mutation (Q190X) in exon 5 also affects both variants I and II and is associated with CNS symptoms (Klein, Grudzien et al. 2007). Two internal splice sites in exon 2 generate variants II and VI, while partial or total retention of introns 1 and 2 generate variants III, IV and V. Splice variants depicted here are named (Lees, Hart et al. 2008) with the nomenclature used by Carlsson et al. in parenthesis. (B) Splice variants identified in the rat HAX1 gene. Variants are named according to Grzybowska et al. and included in parentheses are human splice variants with similarity to the rat transcripts (Fadeel and Grzybowska 2009).

HAX1 expression is quite ubiquitous (Suzuki, Demoliere et al. 1997; Carlsson, van't Hooft et al. 2008), although some variability has been observed across different tissues. Moreover, Rat and human skeletal muscle also displays a relatively high HAX1 expression level; however, surprisingly, in mice the expression observed in this tissue is quite low (Grzybowska, Sarnowska et al. 2006; Hippe, Bylaite et al. 2006; Carlsson, van't Hooft et al. 2008). Immunohistochemical analysis of HAX-1 protein expression in several mouse tissues were not exactly corresponding to the mRNA expression levels (Hippe, Bylaite et al. 2006). Hence, in liver and especially in testis, high amounts of the transcript do not translate into high protein levels. The same phenomenon was observed earlier by immunoblotting in rat tissues (Grzybowska, Sarnowska et al. 2006). This may indicate either that HAX1 mRNA translation is inhibited or that the protein degradation rate is high in these tissues (Fadeel and Grzybowska 2009).

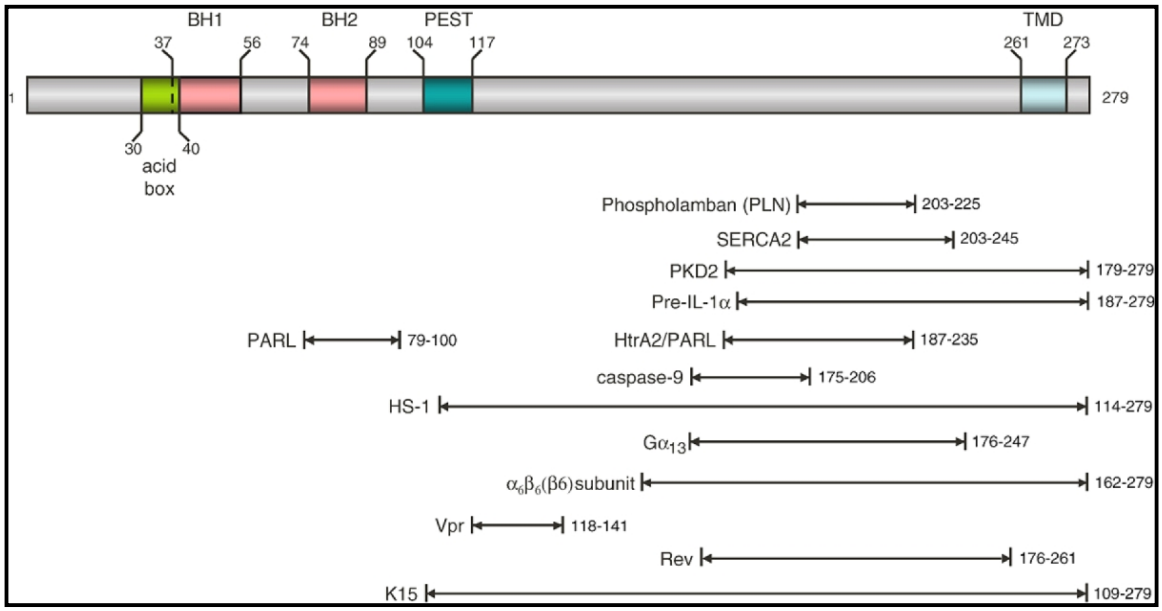


Fig 8: Domain structure of the human HAX-1 protein: The putative Bcl-2 homology (BH) domains and the putative transmembrane domain (TMD), along with the PEST domain are shown. The role of the acid box present at the N-terminus is so far unknown. In addition, it should be noted that the functional significance of the putative BH domains remains to be demonstrated. Regions of interactions with other cellular and viral proteins are indicated and their locations thus suggest that the C-terminal part of HAX-1 is responsible for protein-protein interactions. The amino acid numbering refers to the regions of the HAX-1 protein shown to be involved in protein binding (Fadeel and Grzybowska 2009)

1.7 HAX1 Protein Domains:

HAX1 protein has no significant similarity to many other known proteins (Suzuki, Demoliere et al. 1997). However it has been reported that HAX1 has a weak similarity to Nip3, a Bcl-2 interacting protein, and is considered to contain two BH domains (BH1 and BH2). However Bioinformatic analyses indicate that the reported similarities are poor. HAX-1 also contains a putative PEST sequence (aa 104–117), which suggests rapid and regulated degradation of the protein. There are also some additional interesting features: an acid box (aa 30–41) with unknown function, composed mostly of glutamic and aspartic acids, as well as several recently identified protein-binding regions that are present mostly in the C-terminal part of the protein. In addition, Chao et al. (Chao, Parganas et al. 2008) implied the existence of "HAX-1 homology domain(s)" (HD) that bind to HtrA2, but these regions of HAX-1 appear to overlap more or less with the previously described BH domains (HD2) or with the C-terminal part of the protein (HD1) that is implicated in the binding of numerous other factors.

1.8 HAX1 and its Biological role

1.8.1 Interaction with Viral Encoded Proteins:

After identification as HS1 interacting protein by Yeast two-hybrid screening, and subsequent confirmation of their association in the lysates of Human Burkitt Lymphoma syndrome (Suzuki, Demoliere et al. 1997), several subsequent fishing expeditions have identified HAX-1 as an interacting protein by similar approach, using cellular and viral proteins as "bait"(Fadeel and Grzybowska 2009).

HAX1 was reported to bind to virus encoded proteins namely EBV nuclear antigen leader protein (EBNA-LP) and EBV nuclear antigen 5 (EBNA5) (Kawaguchi, Nakajima et al. 2000; Dufva, Olsson et al. 2001). Studies based on transient transfection and GST pull down experiments using COS7 cells as a model system suggested that EBNA-LP may interact with Bcl-2 via HAX1 (Matsuda, Nakajima et al. 2003).

Kaposi's sarcoma-associated herpesvirus (KSHV) (or human herpesvirus 8, HHV-8) open reading frame (ORF) K15 interacts with HAX-1 through its C-terminal domain. Furthermore, HAX-1 colocalized with K15 in the endoplasmic reticulum and mitochondria. HAX-1 was also reported to block Bax-induced apoptosis in a transient transfection assay in HeLa cervical carcinoma cells. This was the first study indicating that HAX-1 may inhibit apoptosis (Sharp, Wang et al. 2002).

HAX-1 was also found to be interacting with human immunodeficiency virus (HIV)-1-encoded proteins. HAX1 was shown to associate with Vpr, a viral factor that has been implicated in T cell apoptosis through its activation of caspase-3 and caspase-9 and perturbation of the mitochondrial membrane potential. HAX1 was found to be sequestered from mitochondria by Vpr in HeLa cells resulting in cell death (Yedavalli, Shih et al. 2005). Conversely, overexpression of HAX-1 suppressed the proapoptotic activity of Vpr in this model concluding that HAX1 is required for stabilization of mitochondria and Vpr can physically bind to and dislocate HAX-1 from mitochondria leading to destabilization of the mitochondrial membrane potential and apoptosis. It is noteworthy that three different viruses (i.e. EBV, KSHV, and HIV-1) have been found to express factors that bind to HAX-1, after overexpression in model cell lines.

1.8.2 HAX1 as an Anti Apoptotic Molecule:

HAX-1 has emerged as a novel and important player in the cell intrinsic apoptotic pathway of cell death. Several studies have implicated HAX-1 in the regulation of the mitochondrial membrane potential during apoptosis (Sharp, Wang et al. 2002; Cilenti, Soundarapandian et al. 2004; Klein, Grudzien et al. 2007). Klein et al. (Klein, Grudzien et al. 2007) reported recently that peripheral blood neutrophils derived from HAX-1-deficient individuals displayed a higher degree of apoptosis ex vivo when compared to cells from normal donors. In addition, neutrophils from patients but not those from normal controls displayed a pronounced dissipation of the mitochondrial membrane potential in response to the K+ ionophore, valinomycin (Klein, Grudzien et al. 2007). Experiments performed with purified stem cells from patients and controls differentiated into myeloid progenitors in vitro showed that patient cells displayed a significant loss of mitochondrial membrane potential upon treatment with valinomycin. In contrast, affected patients' myeloid progenitor cells in which HAX-1 expression was restored through retroviral gene transduction showed a delayed loss of mitochondrial membrane potential, similar to wild-type cells that were transduced with a control vector. Similar studies showed that there was an exaggerated release of cytochrome c from the mitochondria of myeloid progenitor cells from patients with Kostmann syndrome as compared to healthy controls (Carlsson, Aprikyan et al. 2004)

Recent Studies show that HAX1 interacts with HtrA2 (High temperature requirement protein A2) and PARL (Presenilin-associated-Rhomboid like) within the mitochondria (Cilenti, Soundarapandian et al. 2004; Chao, Parganas et al. 2008). HtrA2, a serine protease is released from mitochondria into the cytosol where it binds the inhibitor of apoptosis proteins (IAPs). This interaction is thought to promote apoptosis by relieving the IAP dependent inhibition of cytosolic caspases (Verhagen, Silke et al. 2002). However, in contrast, genetic evidence provides strong support for an anti-apoptotic role of HtrA2. Hence, mice with a targeted deletion of HtrA2 or point mutations of its protease domain exhibit features of a neurodegenerative disorder with a parkinsonian phenotype with loss of a population of neurons in the striatum (Jones, Datta et al. 2003; Martins, Morrison et al. 2004).

HAX1 forms a complex with the mitochondrial protease PARL, and probably cooperates with it to process HtrA2 into its mature form, which is then released into the intermembrane space and provides protection from apoptosis. Processed HtrA2 was markedly reduced in HAX1 deficient mice (Chao, Parganas et al. 2008). The accumulation of activated Bax in the outer mitochondrial membrane is known to induce cytochrome c release and apoptosis, and the presence of processed HtrA2 prevented the accumulation of Bax in mouse lymphocytes (Chao, Parganas et al. 2008). It will also be interesting to try to reconcile these findings with the previously reported processing of HAX-1 by HtrA2/Omi in mitochondria during apoptosis (Cilenti, Soundarapandian et al. 2004). On the basis of the above mentioned studies, HAX1 and its interacting partners are thus emerging as important regulators of intrinsic (mitochondria-dependent) apoptosis in mammalian cells.

HAX1 mediated inhibition of apoptosis was observed in cardiac myocytes: post mitochondrial repression of caspase 9 (Han, Chen et al. 2006). Through a yeast two-hybrid screening of an adult human heart cDNA library, Han et al. found that HAX-1 associates with caspase-9. Furthermore, enforced overexpression of HAX-1 in cardiac myocytes conferred 30% protection from apoptosis as compared with the control. Direct interaction of cytosolic caspase-9 and the mitochondria-resident protein HAX-1 is difficult to envisage since HAX-1 has been reported to reside within mitochondria (Kasashima, Ohta et al. 2006; Chao, Parganas et al. 2008). On the other hand, re-localization of HAX-1 from mitochondria to the cytosol upon apoptosis stimulation could potentially occur, thus allowing for its physical association with caspase-9. In fact, other investigators have proposed that HAX-1 is a substrate for caspase-3 (Lee, Lee et al. 2008). However, Han et al. (Han, Chen et al. 2006) concluded that HAX-1 is not released into the cytosol in cardiac myocytes and they suggested instead that caspase-9 may translocate to mitochondria during early phases of apoptosis, and may then shuttle back to the cytosol during the execution phase of apoptosis.

1.8.3 HAX1 and its regulatory role in cell migration:

HAX1 is reported to be involved directly and indirectly in cell migration. HAX-1 was shown to interact with the polycystic kidney disease protein PKD2 in HeLa cells

transfected with PKD2 and HAX-1 (Gallagher, Cedzich et al. 2000). Immunofluorescence studies showed that in most cells PKD2 and HAX-1 co-localized in the cytosol, but in some cells the two proteins were also sorted into cellular processes and lamellipodia. These authors also showed that HAX-1 interacts with cortactin, an F-actin-associated protein that closely resembles HS-1. Together, these findings suggest a link between PKD2 and the actin cytoskeleton, possibly mediated via HAX-1 (Fadeel and Grzybowska 2009).

HAX-1 forms a quaternary complex with cortactin, Rac and Gα13, a protein that is primarily involved in the regulation of cell migration (Radhika, Onesime et al. 2004). HAX-1 overexpression was shown to attenuate the formation of actin stress fibers and focal adhesion complexes in Gα13-expressing NIH3T3 cells. Conversely, silencing of endogenous HAX-1 expression with small interfering RNAs drastically reduced Gα13-mediated cell migration. Accordingly, it was proposed that the dynamic association between Gα13 and HAX-1 represents a switch, which is crucial for the regulation of cell motility: formation of the quaternary complex was thus suggested to sequester Gα13 from cell adhesion pathways, thereby shifting the balance to cell protrusion and migration (Radhika, Onesime et al. 2004).

HAX1 was reported to bind the β6 subunit and regulate clathrin-mediated endocytosis of αvβ6 integrins (Ramsay, Keppler et al. 2007). Moreover, reduction of HAX-1 levels by small interfering RNA suppressed αvβ6-dependent migration of oral squamous cell carcinoma cell lines through downregulation of αvβ6 endocytosis via a clathrin mediated pathway. Also, Enhanced expression of HAX1 was observed to lead to oral cancer (Ramsay, Keppler et al. 2007).

The involvement of HAX-1 in the integrin signaling pathway was confirmed by recent proteomics studies identifying HAX-1 and several other proteins as a novel integrin-linked kinase (ILK)-interacting partners (Dobreva, Fielding et al. 2008). However, although actin cytoskeleton remodelling and integrin signaling both constitute important factors in the regulation of cell motility, a direct connection between the two processes is not apparent (Fadeel and Grzybowska 2009).

1.8.4 HAX1 interaction with mRNA:

The two mRNA targets of HAX-1 identified thus far encode for vimentin (Al-Maghrebi, Brule et al. 2002) and DNA polymerase β (POLB) (Sarnowska, Grzybowska et al. 2007). Vimentin is a cytoskeletal protein involved in cell migration. The HAX-1-binding hairpin motif present in the vimentin mRNA was described as a "zip-code" element, directing the transcript into the perinuclear space, which is important for its correct translation, and, in consequence, for proper cell morphology (Morris, Evason et al. 2000). This connection thus suggests a role for HAX-1 in mRNA transport.

The HIV-1 Rev protein facilitates the nuclear export of viral mRNAs containing the Rev response element (RRE). A recent study revealed that HAX-1 interacts with Rev and inhibits its activity in RRE-mediated gene expression (Modem and Reddy 2008). Immunofluorescence studies performed in COS-1 cells further revealed that HAX-1 and Rev are cytoplasmic and nuclear proteins, respectively, when expressed independently. However, in HAX-1 co-expressing cells, Rev translocated from the nucleus to the cytoplasm, where it co-localized with HAX-1 in the cytoplasm (Fadeel and Grzybowska 2009). Overexpression of HAX-1 could interfere with the stability/export of RRE-containing mRNAs and target these RNA transcripts for degradation. It was also proposed that small molecules that mimic HAX-1 and specifically interfere with the pathway shared by Rev (Modem and Reddy 2008) and Vpr (Yedavalli, Shih et al. 2005) could become useful in the treatment of HIV-1-infected AIDS patients (Fadeel and Grzybowska 2009).

1.8.5 HAX1 and its role in human diseases:

Involvement of HAX-1 in apoptosis and cell migration-two processes crucial to carcinogenesis and metastasis suggests that over expression of HAX-1 in cancer is likely to occur, as this could promote cell survival and enhance the invasive potential of malignant cells. Moreover, for comparison, numerous previous studies have demonstrated that anti-apoptotic Bcl-2 and related BH domain-containing proteins are overexpressed in human malignancies. Elevated levels of HAX-1 protein were also detected in melanoma cell lines (Mirmohammadsadegh, Tartler et al. 2003) and oral squamous cell carcinomas (Ramsay, Keppler et al. 2007) by immunoblotting and

immunohistochemistry, respectively. Interestingly, a recent study showed that silencing of HAX-1 expression in a human melanoma cell line resulted in the induction of apoptosis (Li, Feng et al. 2009). Klein et al. (Klein, Grudzien et al. 2007) identified homozygous HAX1 mutations in patients with Kostmann disease, including three patients belonging to the original Swedish kindred described by Kostmann, thus providing definitive proof that Kostmann disease is caused by HAX-1 deficiency. An important lesson that has emerged since the original identification of HAX1 mutations in Kostmann disease patients is that the specific location of the HAX1 mutation may determine the clinical presentation. Carlsson and Fasth (Carlsson and Fasth 2001) first reported the frequent occurrence of neurological deficits in patients belonging to the original family described by Kostmann, and the association between HAX-1-deficiency and CNS abnormalities is further strengthened by the demonstration of extensive loss of neurons in the striatum and cerebellum of HAX1-null mice (Chao, Parganas et al. 2008). Studies from several laboratories now suggest a genotype-phenotype correlation in so far as mutations that affect both HAX1 transcript variants I and II result in neutropenia and neurodevelopmental abnormalities, whereas HAX1 mutations affecting only transcript variant I result in neutropenia without CNS problems (Carlsson, van't Hooft et al. 2008; Germeshausen, Grudzien et al. 2008; Ishikawa, Okada et al. 2008). In the second case the pathological mutation is removed from variant II due to splicing out of the 5′ end of exon 2 (Carlsson, van't Hooft et al. 2008). The location of the HAX1 mutation (W44X) identified in the Kurdish population associated with neutropenia but no CNS symptoms is depicted along with the “classical” mutation (Q190X) identified in the original Swedish kindred described by Kostmann and the alternative mutation (R86X) identified in the Japanese population that affects both variants I and II (Fadeel and Grzybowska 2009).

HAX1 is reported to have a possible role in psoriasis, a chronic inflammatory condition that is believed to be caused by a combination of genetic, immunologic and environmental factors (Mirmohammadsadegh, Tartler et al. 2003) where mRNA based differential display technologies were used to search for disease-associated genes. Upregulation of HAX-1 expression was observed in lesional psoriatic skin at transcriptional and protein levels by northern and western blot analyses. Anti-apoptotic role for HAX-1 in the spontaneously transformed Human epidermal cell line, HaCaT was observed. Antisense-mediated inhibition of HAX-1 expression led to a marked increase

in caspase-3 activation in HaCaT keratinocytes following UVB irradiation (Mirmohammadsadegh, Tartler et al. 2003). The fact that the HAX1 gene is located within the epidermal differentiation complex further implies a regulating function during the epidermal maturation process. HAX-1 has also been implicated in systemic sclerosis, a chronic autoimmune disease characterized by fibrosis of the skin and internal organs, including lungs, kidney, heart and gastrointestinal tract.

HAX-1 interacts with pre-IL-1α in fibroblasts from systemic sclerosis patients, and RNA interference studies indicated that HAX-1 was essential for pre-IL-1α-induced production of IL-6 and procollagen type I synthesis (Kawaguchi, Nishimagi et al. 2006). Moreover, these studies suggested that nuclear localization of pre-IL-1α depends on the binding to HAX-1. It remains possible that additional, unknown protein(s) could also associate with the pre-IL-1α/HAX-1-containing complex and enable nuclear translocation and/or DNA or RNA binding, thus allowing the protein complex to modulate the biological activities of fibroblasts.

HAX-1 associates both with PLN (Vafiadaki, Sanoudou et al. 2007) and SERCA2 (Vafiadaki, Arvanitis et al. 2009), thus implying a potential role for HAX-1 in modulation of Ca2+ homeostasis and providing a further link between Ca2+ homeostasis and cell survival/apoptosis. HAX-1 translocated to the ER in HEK 293 cells upon co-transfection with HAX-1, PLN and SERCA2. However, overexpression of SERCA2 abrogated the pro-survival effects of HAX-1. PLN and SERCA2 thus seem to exert opposing effects on the antiapoptotic effects of HAX-1(Fadeel and Grzybowska 2009).

1.9 Aims of the proposed study.

Hypothesis

HAX1 is a unique protein implicated in a perplexing range of biological functions through its physiological interactions with various cellular and viral factors. The fundamental hypothesis guiding my studies was to get an insight in to the molecular and physiological role played by HAX1 in cell survival.

The Current Thesis had the following specific aims:

1) To identify the potential intracellular interacting proteins of HAX1.

2) To Over express and purify HAX1 for interaction studies

3) To confirm and characterize the interactions

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1CHEMICALS

All chemicals listed below were purchased with highest degree of purity from the following companies:

Acrylamide	Roth, Karlsruhe
Acetone	J.D. Baker
Agarose	Bioline
$AgNO_3$	Sigma, Deisenhofen
Ammonium Chloride	Sigma, Deisenhofen
Ammonium-peroxo-disulfate (APS)	Roth, Karlsruhe
Ampicillin	Sigma Aldrich
Adenosine-5'-triphosphate (ATP)	Boehringer, Mannheim
Adenosin-5'-diphosphate (ADP)	Boehringer, Mannheim
Bacto Yeast Extract	Roth, Karlsruhe
Bacto Tryptone	Roth, Karlsruhe
Bromo-phenol-blue	Roth, Karlsruhe
Calcium Chloride	Sigma, Deisenhofen
Casein peptone	Roth, Karlsruhe
CHAPS	
3[(3-Cholamidopropyl) dimethylammonio]-propanesulfonic acid	Sigma, Deisenhofen
Coomassie Brilliant Blue G-250	Roth, Karlsruhe
Coomassie Brilliant Blue R-250	Roth, Karlsruhe
Deoxynucleoside-5^{I}–triphosphate (dATP, dCTP, dGTP, dTTP)	Fermentas,
Dextrose	Sigma, Deisenhofen
Diethylpyrocarbonate (DEPC)	Fluka, Neu-Ulm
Dimethylformamide (DMF)	Sigma, Deisenhofen
Dimethylsulfoxide (DMSO)	Sigma, Deisenhofen
Dithiothreitole (DTT)	Sigma, Deisenhofen
Ethidium Bromide	Applichem, Damstadt
Ethanol	J.D.Baker
EDTA	Merck

EGTA	Merck
Formaldehyde	Roth, Karlsruhe
Formamide	Roth, Karlsruhe
D-Glucose	Sigma, Deisenhofen
Glycine	Sigma, Deisenhofen
Glycerol	Sigma, Deisenhofen
N-(2-Hydroxyethyl)-1-piperazinoethanosulfonic acid (HEPES)	Gibco
Imidazole	Sigma, Deisenhofen
Kanamycin Sulfate	Sigma, Deisenhofen
L-Adenine Hemisulfate	Sigma, Deisenhofen
L-Arginine HCl	Sigma, Deisenhofen
L-Histidine HCl	Sigma, Deisenhofen
L-Isoleucine	Sigma, Deisenhofen
L-Leucine	Sigma, Deisenhofen
L-Lysine HCl	Sigma, Deisenhofen
L-Methionine	Sigma, Deisenhofen
L-Phenylalanine	Sigma, Deisenhofen
L-Threonine	Sigma, Deisenhofen
L-Tryptophan	Sigma, Deisenhofen
L-Tyrosine	Sigma, Deisenhofen
L-Uracil	Sigma, Deisenhofen
L-Valine	Sigma, Deisenhofen
Lithium acetate	Sigma, Deisenhofen
Leupeptin	Sigma, Deisenhofen
Maltose	Fluka, Neu-Ulm
Manganese-(II) Chloride	Fluka, Neu-Ulm
B-Mercaptoethanol	Sigma, Deisenhofen
3-(N-Morpholino)-propansulfonic acid (MOPS)	Fluka, Neu-Ulm
NP-40	Sigma, Deisenhofen
Neomycinsulfate	Gibco BRL,Eggenstein
PEG 3350	Sigma, Deisenhofen
Penicillin-streptomycin	Gibco BRL,Eggenstein

Penta-His antibody	Qiagen, Dusseldorf
Pepstatin	Sigma, Deisenhofen
Phenol	Roth, Karlsruhe
Phenylmethansulfonsäurefluoride (PMSF)	Sigma, Deisenhofen
Protease peptone	Oxoid, GB
Q-sepharose fast flow	GE Health care
Saccharose	Sigma, Deisenhofen
Sodiumdodecylsulfate (SDS)	Serva, Heidelberg
Sodium azide	Merck, Darmstadt
Sodium Chloride	Merck, Darmstadt
Sodium Carbonate	Merck, Darmstadt
N,N,N^{I},N^{I} – Trtramethylenediamine (TEMED)	Roth, Karlsruhe
N_{α}-p-Tosyl-$_{L}$-Arginin-methyl-ester-hydrochloride (TAME)	Sigma, Deisenhofen
N_{α}-p-Tosyl-$_{L}$-Lysin-chlormethyl-Keton (TLCK)	Sigma, Deisenhofen
N-Tosyl-$_{L}$-Phenylalanin-chlormethyl-ketone (TPCK)	Sigma, Deisenhofen
Triton-X-100	Sigma, Deisenhofen
Tween-20	Sigma, Deisenhofen
Urea	Fluka, Neu-Ulm
X-Gal	Merck, Darmstadt
Yeast Nitrogen Base (YNB)	QBIO gene
3-Amino-1,2,4-triazole	Sigma, Deisenhofen

2.1.2 KITS

Qiagen Plasmid Mini Kit	Qiagen Dusseldorf
Qiagen Maxi plasmid kit	Qiagen Dusseldorf
Protein cutoff membrane filters	Vivascience
Western blot developer	Pierce
Cloning kits	Promega, Roche.
Qiagen DNA Gel Extraction Kit	Qiagen Dusseldorf

2.1.3 Antibiotics

Ampicillin	50mg/ml in water
Kanamycin	50mg/ml in water

Penicillin/Streptomycin	20mg/ml in water
G418	20mg/ml in water

2.1.4 Resins for purification

Ni super fast flow resin	Qiagen
Glutathione sepharose 4fast flow	GE health care
Protein G sepharose	GE health care
HighLoad Sephadex 200	GE health care

2.1.5 Instruments

FACS Calibur (BD-Pharmingen, SD, CA)

Cell sorter Moflow (DAKO Cytomation, Denmark)

ELISA reader (Molecular Devices, MWG-Biotech, Germany)

Microscope (confocal laser scanning microscope TCS-SP, Leica, Germany)

Agarose gel chamber (Biorad, Germany)

Spectrophotometer (Eppendorf, Germany)

Master cycler gradient (Eppendorf, Germany)

Western blot apparatus (Bio-Rad, Germany)

Centrifuge (Sorval; Eppendorf, Germany)

ChemiDoc (Bio-Rad, Germany)

Veritas™ Microplate Luminometer (Turner Biosystems)

2.1.6 Cloning and Expression Vectors

2.1.6.1 Subcloning and Gateway cloning entry vectors:

Vector	Type of cloning	Resistance	Company
pGEM-T	Restriction digestion	Ampicillin	Promega
pENTR™/D-TOPO®	Gateway cloning	Kanamycin	Invitrogen

2.1.6.2 *E.coli* cloning vectors:

Vectors	Resistance	Company
pET-24(d)	Kanamycin	Novagen
pGEX4T1	Ampicillin	GE Health care

2.1.6.3 Yeast cloning vectors:

vectors	Fusion	Fusion	Selection	Resistance
pGBKT7	DNA/Bait	C-myc	*TRP1*	Kanamycin
pDEST GAD	AD/ Prey	HA	*LEU2*	Ampicillin
pGADT7	AD/ Prey	HA	*LEU2*	Ampicillin

2.1.6.4 *Dictyostelium discoideum* Cloning vectors:

Vector	Fusion	Resistance	Cleavage site
pDXA M765 MCS	Catalytic domain of Myosin	Ampicillin/Neomycin	Thrombin
pDXA M765 TEV	Catalytic domain of Myosin	Ampicillin/Neomycin	TEV

2.1.6.5 Mammalian Cloning Vectors:

Vectors	Resistance	Company
pCDNA3	Ampicillin	Invitrogen
pCDNA3.1(+)	Ampicillin	Invitrogen
pEGFPC1	Kanamycin	Clontech
pEGFPC3	Kanamycin	Clontech
pDEST27	Ampicillin	Invitrogen
pDEST26	Ampicillin	Invitrogen
pDEST CMyc	Ampicillin	

2.1.6.6 Retroviral cloning vectors:

Vector	Resistance	Surrogate-Marker
pMMP IRES GFP	Ampicillin	GFP
pMMP IRES mCD24^{+}	Ampicillin	mCD24^{+}
pQCXIX	Ampicillin	___

2.1.6.7 E.coli Expression constructs:

Cloning strategy for expression constructs:

Clones	Vector	Restriction site	Resistance
mHAX1-HIS	pET-24d	BspH1/Nco1 - HindIII	Kanamycin
GST-mHAX1	pGEX4T-1	BamH1-EcoR1	Ampicillin
HHAX1-HIS	pET-24d	Nco1-EcoR1	Kanamycin
GST-HHAX1	pGEX4T-1	BamH1-EcoR1	Ampicillin

2.1.6.8 Mammalian system expression constructs:

Cloning strategy for expression constructs

Clones	Vector	Restriction sites	Resistance
pDEST27-mHAX1	pDEST27	Gateway cloning	Ampicillin
pDEST27-mBif-1	pDEST27	Gateway cloning	Ampicillin
pDEST27-mSerpin b1a	pDEST27	Gateway cloning	Ampicillin
pDEST 26-mBif-1	pDEST26	Gateway cloning	Ampicillin
pDEST26-mSerpin b1a	pDEST26	Gateway cloning	Ampicillin
pDEST Cmyc-mBif-1	pDEST CMyc	Gateway cloning	Ampicillin
pDEST Cmyc-mSerpin b1a	pDEST CMyc	Gateway cloning	Ampicillin
pCDNA3-mHAX1	pCDNA3	BamH1-Not1	Ampicillin
pCDNA3-HHAX1	pCDNA3	BamH1-Not1	Ampicillin
pCDNA3.1(+) -mHAX1	pCDNA3.1(+)	BamH1-Not1	Ampicillin
pCDNA3.1(+) -HHAX1	pCDNA3.1(+)	BamH1-Not1	Ampicillin
pEGFPC3-mHAX1	pEGFP-C3	SacII-BamH1	Kanamycin

2.1.6.9 Deletion mutants of HHAX1: These mutants were received from Dr Claudia's lab.

Clones	Vector	Restriction sites	Resistance
pEGFPC1-HHAX1(1-279)	pEGFP-C1	Bgl2-Sal1	Kanamycin
pEGFPC1-HHAX1(1-190)	pEGFP-C1	Bgl2-Sal1	Kanamycin
pEGFPC1-HHAX1(190-279)	pEGFP-C1	Bgl2-Sal1	Kanamycin
pEGFPC1-HHAX1(179-279)	pEGFP-C1	Bgl2-Sal1	Kanamycin
pEGFPC1-HHAX1(129-279)	pEGFP-C1	Bgl2-Sal1	Kanamycin
pEGFPC1-HHAX1(179-234)	pEGFP-C1	Bgl2-Sal1	Kanamycin
pCS2MT-HHAX1(1-279)	pCS2-MT	EcoR1-Sal1/Xho1	Ampicillin
pCS2MT-HHAX1(1-234)	pCS2-MT	EcoR1-Sal1/Xho1	Ampicillin
pCS2MT-HHAX1(1-190)	pCS2-MT	EcoR1-Sal1/Xho1	Ampicillin
pCS2MT-HHAX1(1-44)	pCS2-MT	EcoR1-Sal1/Xho1	Ampicillin

2.1.6.10 Yeast Expression constructs:

Clones	Fusion	Selection	Resistance
pGBKT7-m HAX1	DNA-BD/m HAX1, C-myc	*TRP1*	Kanamycin
pDEST GAD-m Bif-1	AD/m Bif-1, HA	*LEU2*	Ampicillin
pDEST GAD-m Serpin b1a	AD/m Serpin b1a, HA	*LEU2*	Ampicillin

Yeast Control vectors	Fusion	Selection	Resistance
pGADT7-SV40T	AD/T-Antigen,HA	*LEU2*	Ampicillin
pGBKT7-P53	DNA-BD/P53, C-myc	*TRP1*	Kanamycin

2.1.6.11 *Dictyostelium discoideum* expression constructs*:*

Cloning strategy for expression constructs

Clones	Vector	Restriction sites	Resistance
pDXA M765 MCS-mHAX1	pDXA M765 MCS	Sac1-Not1	Ampicillin
pDXA M765 TEV-mHAX1	pDXA M765 TEV	Sac1-Not1	Ampicillin
pDXA M765 MCS-HHAX1	pDXA M765 MCS	Sac1-Not1	Ampicillin
pDXA M765 TEV-HHAX1	pDXA M765 TEV	Sac1-Not1	Ampicillin

2.1.6.12 Retroviral expression constructs:

Cloning strategy for expression constructs

Clones	Vector	Restriction sites
pMMP IRES GFP-mHAX1	pMMP IRES GFP	Nco1/BspH1-BamH1
pMMP IRES GFP-C Myc-mHAX1	pMMP IRES GFP	Nco1/BspH1-BamH1
pMMP IRES mCD24^{+}-mHAX1	pMMP IRES mCD24^{+}	Nco1/BspH1-BamH1
pQCXIX-YFP-LC3	pQCXIX	

The pQCXIX-YFP-LC3 was a kind gift of Prof Kubicka, Dept of Gastroenterology, MHH.

2.1.7 List of oligonucleotides (5' to 3')

Primers used for the generation of full-length* H.HAX1 *DNA constructs:

EcoR1 HHAX pGEX4T1 (sense) GAA TTC ATG AGC CTC TTT GAT

BamH1pGEX H HAX (sense) GGA TCC ATG AGC CTC TTT GAT

EcoR1 pGEX H HAX (Antisense) GAA TTC CTA CCG GGA CCG GAA

Sac1 Dicty H HAX (sense) GAG CTC ATG TGC CTC TTT GAT CTC

Not1 Dicty H HAX (Antisense) GCG GCC GCC TAC CGG GAC CGG AAC

pDEST HHAX (sense) CACC ATG AGC CTC TTT GAT

pDEST HHAX (Antisense) CTA CCG GGA CCG GAA CCA

Primers used for the generation of full-length* m.HAX1 *DNA constructs

BspH1 mHAX cDNA (sense) TCA TGA GCG TCT TTG ATC TTT TC

BamH1 mHAX cDNA (Antisense) GGA TCC TAC CGG GAC CGG AAC CAA

HindIII pET24d (Antisense) AAG CTT TCG GGA CCG AAA

Sma1 pGEX4T1 (sense) CCC GGG TAT GAG CGT CTT T

BamH1 pGEX mHAX (sense) GGA TCC ATG AGC GTC TTT GAT

Sac1 Dicty mHAX (sense) GAG CTC ATG TGC TCT TTG ATC

Not1 Dicty mHAX (Antisense) GCG GCC GCC TAT CGG GAC CGA

pDEST mHAX (sense) CACC ATG AGC GTC TTT GAT

pDEST mHAX (Antisense) CTA TCG GGA CCG AAA CCA

Primers used for the generation of full-length* m.Bif-1 *DNA constructs

pDEST mEndophilinb1a (sense) CACC ATG AAC ATC ATG GAT

pDEST mEndophilin b1a (Antisense) TTA ATT TAG AAG TTC TAA

Primers used for the generation of full-length* m.Serpinb1a *DNA constructs

pDEST mSerpinb1a (sense) CACC ATG GAG CAG CTG AGT

pDEST mSerpinb1a (Antisense) CTA TCG GGA ACA AAC CCT

2.1.8 Enzymes for molecular biology

Calf Intestinal alkaline Phosphatase:	Fermentas Life Sciences (Cat no EFO341)
T4 DNA-ligase:	Fermentas Lifesciences (Cat no EL0011)
Hi-fed DNA polymerase:	Roche

All restriction digestion enzymes used were purchased from Fermentas Lifesciences.

2.1.9 Antibodies

2.1.9.1 Primary Antibodies

Purified Mouse Anti-HAX-1:	BD Transduction Laboratories™
Monoclonal Antibody to Human Bif-1:	Imgenex
Beclin1 Antibody:	Cell Signalling
Polyclonal Antibody to UVRAG:	Imgenex
Monoclonal Anti-BAX, clone 6A7:	SIGMA
PI3 Kinase Class III (D4E2) Rabbit Mab:	Cell Signalling
BAX (N-20):	Santa Cruz Biotechnology
Phospho-Bcl-2 (Ser70) (5H2) Rabbit mAb:	Cell signaling
Mouse Anti Phospho Tyrosine:	BD Transduction laboratories™

2.1.9.2 Secondary antibodies:

Goat anti Mouse-HRP:	BD Transduction laboratories
Goat anti Rabbit-HRP:	Cell Signalling
Goat anti Mouse-Alexa 546:	Molecular probes
Goat anti Rabbit-Alexa 488:	Molecular probes
Goat anti Rabbit-Alexa 633:	Molecular probes

Goat anti Mouse-Alexa 594: Molecular probes

2.1.10 Standards

2.1.10.1 Size of DNA molecular weight marker (DNA *ladder*-marker): (Peqlab)

Size of DNA in Base pairs: (100bp ladder)

3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100

Size of DNA in Kb: (1Kb ladder)

10, 8, 6, 5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.75, 0.5, 0.25.

2.1.10.2 Molecular weight marker for proteins:

PageRuler™ Prestained Protein Ladder: size of peptide in KD:

170, 130, 100, 70, 55, 40, 35, 25, 15, 10 (Fermentas Lifesciences)

2.1.11 General buffers and stock solutions

TE

40mM Tris/HCl (pH 8.0)

1mM EDTA

TAE

40mM Tris (pH8.0)

20mM Acetic acid

2mM EDTA

TBST

20mM Tris/HCl (pH7.6)

137mM NaCl

0.05% Tween-20

PBS (phosphate buffered saline)

80mM Na_2HPO_4 (pH 7.4)

20mM NaH_2PO_4

100mM NaCl

TBS (Tris buffered saline)

20mM Tris/HCl (pH7.6)

137mM NaCl

20 X SSC

3M NaCl

0.3M tri-sodium-citrate

PBS buffer (PBST)

8 g/l NaCl

0.2 g/l KCl

1.44 g/l Na_2HPO_4

0.24 g/l KH_2PO_4

(0.05 % Tween-20)

Adjust pH to 7.4 with HCl, 1 liter water solution.

2.1.12 Materials for the cultivation of *Escherechia coli*

2.1.12.1 *E.coli* strains

1) *E.coli* XL1-Blue

2) *E.coli* BL21 PLYS S, DE3

3) *E.coli* Rosetta

4) *E.coli* XL Gold

2.1.12.2 Media and Buffers for the cultivation of *E.coli*

LB-medium

10 g/L Tryptone (or peptone)

5 g/L Yeast extract

10 g/L NaCl

1 liter water solution, autoclave 30min at 121°C

50 µg/ml Ampicillin

50 µg/ml Kanamycin

LB-Agar plates

Add 15g/L Bacto agar to LB-medium and autoclave

SOB-medium

20 g/l Bacto Tryptone

5 g/l Bacto Hefextract

10 mM NaCl

2.5 mM KCl

30 min at 121°C Autoclaved; 1% sterile 1M $MgCl_2$ and 1M $MgSO_4$ were added to the medium.

SOC-medium

0.4 % (w/v) glucose in SOB-medium

Cryopreservation of bacteria

500µl of overnight culture were added to 250µl of glycerol (87%) in a cryotube and stored at-80 ^{o}C.

2.1.13 Materials for culturing Yeast *Saccharomyces cerevisiae*

2.1.13.1 Media

SD DROPOUT MEDIUM (- Tryptophan)	
10X SD	5.0ml
20% Glucose	5.0ml
100X Amino Acid mixture	0.5ml
100X Adenine	0.5ml
100X Leucine	0.5ml
100X Histidine	0.5ml
Sterile DH2O	38.0ml

2X YPDA MEDIUM	
Peptone	3.0g
Yeast extract	1.5g
DH_20	133.5 ml

The above solution has to be autoclaved at 121^{o}C for 30 min. To this 15 ml of sterile 20% Glucose and 1.5 ml of sterile 100X Adenine solution is to be added before use.

2.1.13.2 Solutions for Yeast Transformation

1.0 M LiAc:

Dissolved 5.1 g of LiAc dihydrate in 50 mL of water; autoclaved at 121^{o}C for 15 min and store at room temperature.

PEG MW 3350 (50% [w/v]):

Dissolved 50 g of PEG 3350 in 30 mL of distilled/deionized water in a 150-mL beaker on a stirring hot plate with medium heat. Allowed the solution to cool to room temperature and made up the volume to 100 mL in a 100-mL measuring cylinder. The solution was mixed thoroughly and autoclaved at 121^{o}C for 15 min.

Single-stranded carrier DNA (2.0 mg/mL):

Dissolved 10 mg/ml of Herring testes carrier DNA in 4 mL of TE buffer (10 m*M* Tris-HCl, 1 m*M* Na2 EDTA, pH 8.0) with constant stirring at 4°C overnight. Stored at –20°C in five 1 mL aliquots. Prior to use, the carrier DNA was denatured in a boiling water bath for 5 min and immediately chilled on ice.

2.1.13.3 Solutions for X-Gal Colony lift filter assay:

Z Buffer

$Na_2HPO_4.7H_2O$	16.1 g/L
$NaH_2PO_4.7H_2O$	5.5 g/L
KCl	0.75 g/L
$Mg_2SO_4.7H_2O$	0.246g/L

Adjust the pH to 7.0. Autoclave at 121^{o}C, 30 min.

Store at room temperature.

X-Gal stock solution:

Dissolve 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in N,N-Dimethyl Formamide (DMF) at a concentration of 20 mg/ml. store in dark at -20^{o}C.

Z Buffer / X-gal solution:

Z buffer	100.00 ml
B-Mercaptoethanol	0.27 ml
X-Gal stock solution	1.67 ml

2.1.14 Materials for the cultivation of *D.discoideum*

2.1.14.1 Media and Buffers

HL5c-medium

10	g/l	protease-peptone
5	g/l	Bacto Yeast extract
10	g/l	Glucose
1.2	g/l	KH_2PO_4 (anhydrous)

SM-AGAR

10	g/l	Glucose
10	g/l	BactoPepton (Difco)
10	g/l	Bacto Yeast Extract
1	g/l	$Mg_2SO_4.7H_2O$

0.4	g/l	Na_2HPO_4 (anhydrous)
1.9	g/l	KH_2PO_4 (anhydrous)
1	g/l	K_2HPO_4 (anhydrous)
20	g/l	Bacto Agar (Difco)

Adjust pH to 6.6 with 2N NaOH, autoclave at 121°C, 30 min.

Adjust pH to 6.4 with 2N NaOH, autoclave at 121°C, 30 min.

EP-Buffer (electroporation buffer)

10	mM	sodiumphosphate (pH6.3)
50	mM	Sucrose

MES-AGAR

20	g/l	Agar in MES-Buffer

Bonner's Solution

0.6	g	Nacl
0.75	g	KCl
0.3	g	$CaCl_2$

1 liter water solution,
autoclave for 30 min at 121°C

MES-Buffer

20	mM	MES (pH 6.8)
0.2	mM	$CaCl_2$
2.0	mM	$MgCl_2$

Adjust pH to 6.8 with 2N NaOH, autoclave for 30 min at 121°C.

***D.discoideum* cell-lines**

Orf^+ cells (Manstein et al., 1989)

2.1.15 Mammalian cell culture system

2.1.15.1 Cell lines

293

The 293 cells are human embryonic kidney cells that have been transformed with adenovirus 5 DNA. This is a hypotriploid cell line, growing as an adherent monolayer in DMEM supplemented medium.

293T

These are 293 derived cells but with an additional SV40 large T-antigen. They grow as an adherent monolayer in DMEM supplemented medium.

293gpg

This cell line has been made in the lab of R. mulligan. It is a stable 293 derived packaging cell line for the production of high titer retroviruses that are Vesicular Stomatitis Virus G (VSVG) pseudotyped. 293gpg cells produce the retroviral gag-pol polyprotein under the control of the tetracycline-regulatable gene expression system (tet^R/VP 16 transactivator – tet^O promoter system).

NIH 3T3

The NIH 3T3 cells are established from mouse fibroblasts after several rounds of serial subcloning. This cell line grows in DMEM supplemented medium as an adherent monolayer.

Jurkat

The Jurkat cell line was established from the pheripheral blood of a 14 year old boy with T-cell lymphoblastic leukemia by Schneider etal, and was originally designated JM^{78}. The cells grow in suspension culture in RPMI supplemented medium.

HeLa

This cell line was derived from cervical cancer cells taken from **He**nrietta **La**cks, who died from her cancer. These cells grow as an adherent monolayer in complete DMEM medium.

A549

A549 cells are carcinomic human alveolar basal epithelial cells. The human alveolar epithelial cell line A549 may be anchored or suspended in a solution *in vitro*. These cells were cultured as an adherent monolayer in complete DMEM medium.

2.1.15.2 Solutions required for cell culture.

RPMI-medium: RPMI, containing 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml glutamine, and 10 μM 2- Mercaptoethanol

IMDM-medium: IMDM, containing 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml glutamine, and 10 µM 2-Mercaptoethanol

DMEM-medium: DMEM, containing 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml glutamine

2.1.15.3 Reagents for the production of retroviruses

(Calcium phosphate transfection)

Calcium chloride

2M calcium chloride solution was prepared in ddH_2O

Low TE

Low TE buffer was made by mixing Tris- HCl pH 7.5 at a final concentration of 1mM with EDTA pH 8.0 at a final concentration of 0.05mM. The solution was filtered

2X HBS

A solution was prepared containing 0.3M NaCl, 50mM Hepes and 1.5mM NaH_2PO_4. pH was adjusted to 7.2. The solution was filtered.

2.1.15.4 Reagents for retrovirus transduction

Polybrene

Polybrene from Sigma-Aldrich was used at a final concentration of 8µg/ml for retroviral transduction.

2.1.15.5 Reagents for Transient cell transfection:

PEI (Polyethylenimine, Sigma): stock solution (1µg/ml pH7.0)

1g of PEI was dissolved in 1 liter of sterile dH_2O and the pH was adjusted to 7.0. The solution was then filter sterilized and stored in 1ml aliquots at -80 ^oC.

2.2 Methods

2.2.1 Molecular biological methods

2.2.1.1 Isolation of plasmid DNA

Plasmid DNA was isolated using either QIAprep spin Miniprep Kit from Qiagen or Nucleobond AX Maxiprep from Machery &Nagel following the manufacturer's instructions.

2.2.1.2 Enzymatic modification of DNA

Digestion with restriction endonucleases, dephosphorylation with alkaline phosphatase and ligations were performed with enzymes and their respective buffers purchased from Fermentas Life Sciences as per the manufacturer's instructions.

2.2.1.3 Amplification of DNA

Standard PCR reactions contained the following components: 10ng of DNA, two specific primers (0.5 µM each), 0.2 µM dNTPs, 5% DMSO, 1 µl of 10 X polymerase buffer with $MgCl_2$, 0.25U taq/pfu polymerase and PCR grade H_20 to a final volume of 10 µl in a 0.2ml PCR tube in an Eppendorf Thermal cycler. The length of denaturation, annealing and elongation were dependent on the characteristics of the DNA template (e.g. Plasmid DNA or Genomic DNA, GC-content of the fragment).

The optimal annealing temperature of primers was determined experimentally starting with a value calculated according to the formula 4X (G/C) + 2X (A/T) -5 and the length of the amplified PCR product.

2.2.1.4 Preparation of RNA

Total RNA was prepared using the RNA Isolation Kit from Stratagene, following the manufacturer's instructions. DNA digestion was carried out during the RNA purification step using the RNase free DNase of Qiagen. Determination of purity and concentration of samples was carried out by spectrometric analysis..

2.2.1.5 First Strand cDNA sysnthesis

cDNA synthesis was performed in a thermal cycler using 1-1.5 µg of total RNA and 10 picomoles of specific reverse primer or oligo (dT) primer with H20 to a final volume of 11.5 µl in a 0.2ml PCR tube. Denaturation was done at 65^{o}C for 10 min. Subsequently the following components were added to a final volume of 20 µl:1 µl Expand buffer, 10mM DTT, 2.5mM dNTPs, 20 Units RNase inhibitor and 50 Units Expand reverse transcriptase. The samples were incubated at 42 ^{o}C for 1 hr and stored at -20C. For the PCR, 6-10 µl of cDNA were mixed with two specific primers (10 picomoles each), 200 µM dNTPs, 1 X polymerase buffer, 3mM $MgCl_2$, 5 U of Taq/Pfu polymerase and filled up with H20 to a final volume of 50 µl. Cycling conditions were determined experimentally.

2.2.1.6 Electrophoresis of DNA and Extraction from Agarose gels

DNA loading buffer (6X) was added to plasmid DNA or DNA fragments and the DNA was separated electrophoretically in 0.5-1.5% agarose gels in TAE running buffer. DNA fragments were purified using the QIA quick Gel Extraction Kit from Qiagen according to the manufacturers' instructions.

6X DNA sample buffer:		**1X TAE Buffer**
50% (v/v)	Glycerol	40 mM Tris-Acetate
0.25% (W/v)	Bromophenol Blue	1 mM EDTA
0.25% (W/v)	Xylene Cyanol	

Dissolved in 1 X TAE buffer

2.2.1.7 pGEMT cloning of PCR products

cDNA generated by PCR usually cannot be used for direct ligation in expression vectors. pGEMT cloning (Promega) allows simple subcloning of PCR products for the amplification in *E.coli*. Taq polymerase adds a single 3'-A overhang to the ends of the PCR product. These fragments are then subcloned into a linearized vector with complementary single, 3'-T overhangs to which topoisomerase I is covalently bound.

Topoisomerase I mediates the ligation of PCR product and vector. 0.5 to 2 µl of PCR product is ligated into pGEM®-T as recommended by the manufacturer.

2.2.1.8 5'–dephosphorylation of DNA fragments

Calf intestinal Alkaline Phosphatase was used to delete the 5' –Phosphate group of vector and insert respectively to avoid religation. The fragments were incubated at 37 ^{o}C for 30 min and the phosphatase was inactivated at 65 ^{o}C for 15 min.

2.2.1.9 Ligation of DNA fragments

DNA fragments can be joined covalently using T4 DNA-ligase. The fragments were mixed with 1µl of 10X ligase buffer and 1 µl of Ligase (Fermentas Life sciences) in a total volume of 10 µl. Ligation was carried out either at 16 ^{o}C overnight or at RT for 2hr.

2.2.1.10 Gateway Cloning

It is based on the nature of site-specific recombination, the integrative and excisive recombination reactions between chromosomes of Bacteriophage lambda and *E. coli* bacterium. The integrative recombination is catalyzed by Int (integrase) and IHF (Integration Host Factor). The recombination between *att*B and *att*P sites results in *att*L and *att*R sites that flank the integrated lambda DNA. Three proteins (IHF, Int and Xis) are required for the excisive recombination. The *att*L and *att*R sites located at both sides of the inserted phage genome DNA recombine site-specifically during the excision event, reforming the *att*P site in lambda and the *att*B site in the *E.coli* chromosome. These processes, performed in vitro, are the basis of the Gateway® Cloning Technology.

2.2.1.11 pENTR-D/TOPO entry cloning:

Blunt end PCR fragment with CACC at the 5' of the gene of interest was created using specific primers and cloned into pENTR™/D-TOPO® vector as per the manufacturer's instructions. The clone was confirmed by sequencing

2.2.1.12 LR recombination:

LR recombination reaction using Gateway® LR Clonase™ II enzyme mix was performed between the entry clone and the gateway destination vector as per the manufacturer's protocol and the clone was confirmed by sequencing and western blot.

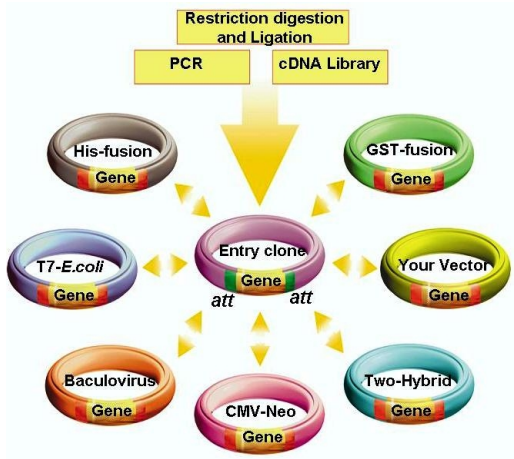


Fig: The gene of interest can be moved into an Entry Vector via PCR, restriction endonuclease digestion and ligation, or site-specific recombination from a cDNA library constructed in a GATEWAY-compatible vector. A gene in the Entry Clone can then be transferred simultaneously into Destination Vectors.

2.2.1.13 Preparation of Chemically competent E.coli cells

200ml of LB-medium without antibiotic was inoculated with an overnight culture of *E.coli* and shaken at 37 °C. At and OD_{600} of 0.3-0.4, It was transferred to centrifuge tubes and kept on ice for 15 min before centrifugation at 3000g, 4 °C for 10 min. Bacteria was resuspended in 60 ml of buffer 1, put on ice for another 15 min and centrifuged as above. The pellet was resuspended in 4 ml of buffer2, aliquoted, flash frozen in liquid nitrogen and stored at -80 °C.

Buffer 1

KOAc, pH 7.5, 30mM

RbCl 12g/L

$CaCl_2.2H_2O$ 50mM

$MnCl_2.4H_2O$ 50mM

Glycerol 150g/L

Adjust pH with 0.2M acetic acid to pH 5.8

Buffer 2

MOPS/NaOH, pH 6.8 10mM

RbCl 12g/L

$CaCl_2.2H_2O$ 11g/L

Glycerol 150g/L

2.2.1.14 Preparation of Electro competent bacteria.

200 ml of LB medium without antibiotics was inoculated with 1/100 volume of fresh overnight culture of bacteria. The cells were cultured at 37^{o}C, shaking at 180 rpm until they reach an ABS_{600} of 0.5 to 1.0. The culture flask was chilled on ice for 15 to 30 minutes and centrifuged at 3750 rpm for 20 min at 4^{o}C. The supernatant was discarded and the pellet was resuspended in 200 ml of sterile cold 15% glycerol. The cells were pelleted and washed twice more with 100 ml and 4ml of cold 15% glycerol respectively. The cells were then centrifuged once again and resuspended in 0.6 ml sterile cold 15% glycerol. 90µl aliquots of were frozen over chilled ethanol in sterile eppendorf tubes and stored at -70C.

2.2.1.15 Transformation of bacteria

2.2.1.15.1 Heat shock protocol:

One aliquot of competent bacteria was thawed and transferred to a 1.5ml reaction tube and kept on ice for 10 min. 200 ng DNA was added to the bacteria and kept on ice for 30 min. Heat shock was performed in a heating block at 42 ^{o}C for 90 sec and instantly placed on ice for another 2 min. 200 µl of SOC medium was added and incubated at 37 ^{o}C for 1 hr. The bacteria was platted on a prewarmed agar-plate with the appropriate antibiotic and incubated overnight.

2.2.1.15.2 Electroporation of bacteria

Competent bacteria, 0.2µl E/P chamber, holder and eppendorfs were pre-cooled on ice. 2 µl of DNA, 40 µl of electrocompetent bacteria were mixed and incubated for 30 min on ice. The E/P unit was set to 2.5 Kvolts, 200 Ω and a capacitance of 25 µFD. After the pulsing, 350 µl of SOC medium was added and the cells were incubated at 37^{o}C for 1hr, plated and grown overnight at 37^{o}C.

2.2.2 Yeast Two hybridization Screen

2.2.2.1 Principle

This system is based on the functional reconstitution of an intact transcription factor that activates reporter gene expression, which can be efficiently selected for in yeast. As indicated by the name, the yeast two-hybrid system utilizes two hybrid proteins: The bait protein (X) fused to the DNA binding domain (DB) and the prey protein (Y) fused to the transcription activation domain (AD) of a transcription factor. In our system, we used the yeast transcription factor Gal4. When X and Y proteins are co-expressed in yeast and can physically interact with each other, the functional transcription factor is reconstituted activating the expression of a set of reporter genes

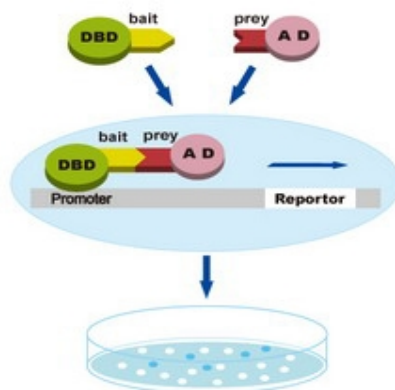


Fig 9: Yeast Two Hybrid Principle

2.2.2.2 Yeast Transformation

Cultured AH109 Yeast cells in 50 ml 2X YPDA medium (2X YPDA, 20% Glucose, 100X Adenine) at 29-30°C over night in a shaker at 200rpm. Harvested the cells when the OD 600 reached 0.6 by centrifugation at 3500 rpm for 5 min at 4°C. The cells were washed thrice with 50 ml of Ice cold Sterile DH_2O. They were then resuspended in 1 ml of Ice cold DH_2O by vortexing in a sterile 1.5 ml Eppendorf tube. 100 µl of the suspension was taken in a fresh Eppendorf tube and centrifuged at maximum speed for 20 seconds and the following transformation mixture was added to the cell pellet.

50% PEG (sterile)	240 µl per reaction
1M LiAc (sterile)	36 µl per reaction
Herring single strand DNA	50 µl per reaction

326 µl of the above transformation mixture was added per each transformation reaction. To this 2 µg of Bait Plasmid DNA was added and the tube was vortexed vigorously to make an even cell suspension. The reaction tubes were then incubated at 42°C for 90 minutes with vigorous shaking. The tubes were then centrifuged at maximum speed for 20 to 30 sec (Room temp) and the pellet was resuspended in 200 µl of sterile DH_2O.

2.2.2.3 Transactivation test

After transformation of Yeast AH109 cells with the Bait plasmid, the cells were plated on **SD Agar ΔHT** (Histidine, Tryptophan), **SD Agar ΔAT** (Adenine, Tryptophan) and **SD Agar ΔAHT** (Adenine, Histidine and Tryptophan) to check for transactivation of the bait protein. Colonies should grow only on **SD Agar ΔHT** plate

2.2.2.4 Protein expression

To check for the protein expression, colonies from **SD Agar ΔHT** plate were picked and cultured in 3ml SD dropout medium with out Tryptophan for 24 hrs. 1ml of culture was centrifuged for 1 min in an eppendorf centrifuge at full speed. The cell pellet was resuspended in 150µl of YEX lysis buffer (1.85M NaOH, 7.5% β-Mercaptoethanol) and incubated on ice for 10 min. 150 µl of cold 50% Trichloroacetic acid was added and incubated on ice for another 10 min. The extracts were centrifuged for 5 min at full speed and resuspended in SDS loading buffer and boiled for 3min at 95C and centrifuged at full speed for 5 min. The supernatant was loaded on 10% SDS page gel and detected for the expression of bait protein.

2.2.2.5 Library transformation-Two Hybrid Screen

The clone with the Bait plasmid confirmed for transactivation was inoculated in 50 ml of **SD ΔTRP** medium under shaking at 250 rpm over night at 30C. Next morning 1ml of Pretransformed 17 day mEmbryo in Yeast Y187 was thawed and inoculated in 50 ml of 2x YPDA medium and cultured for 5 hrs at 250 rpm at 30C. The cells were then centrifuged at 1000g for 5 min. The pellet was resuspended in 4 ml of YPDA medium with 15%glycerol. 1 ml of this suspension is used for mating. 10 µl of this suspension was used for titration on **SD Δ Leucine** plates**.**

The over night culture of the bait plasmid was centrifuged at 1000g for 5 min at RT. The pellet was resuspended in 50 ml of 2x YPDA and 1ml aliquot of the library was added and mixed. This mixture was placed in a sterile 2 liter conical flask and allowed to culture at 40 rpm for 24 hrs. The cells were centrifuged for 10 min at 1000g.The pellet was resuspended in 10 ml of 0.5 x YPDA medium.10 µl of this suspension was used to make serial dilutions of 1:100, 1:1000, 1:10,000 in 0.5x YPDA and 100 µl of each dilution was stroked on **SD Δ LT** in duplicates. The remaining suspension was streaked on 50 **SD Δ AHLT** plates (15 cm Dia) with 200 µl per plate. The plates were incubated at 30C for 5 days for the positive clones to express.

2.2.2.6 X-Gal colony Lift filter assay

The colonies were replica platted on **SD Δ AHLT** agar plates and incubated for 3 to 8 days at 30C. The colonies were streaked on fresh plates and incubated for 2 to 4 days. For each plate to be assayed, a sterile Whatmann # 5 filter paper was soaked in 5 ml of Z buffer/X-gal buffer in a 100-150 mm plate. Using forceps a clean, dry filter was placed over the surface of the plate with colonies to be assayed. The filter was gently rubbed with the side of forceps to help the colonies cling to the filter. The orientation of the filter was marked and carefully lifted off the agar plate with forceps, transferred (colonies facing up) to a pool of liquid nitrogen completely submerging it for 10 sec. The filter was removed after it was found to be completely frozen and allowed to thaw at room temperature. The filter was very carefully placed, colony side up on the presoaked filter making sure that there were no air bubbles trapped in between. The filters were incubated in dark at RT and checked periodically for the appearance of blue colonies. By aligning the filter to the agar plate using the orientation marks the corresponding blue colonies were cultured from the original plates for further analysis.

2.2.2.7 Isolation of Yeast plasmids:

The positive blue colonies were inoculated into 3 ml of **SD Δ Leu** medium and incubated at 30C overnight with shaking at 230-250 rpm. The cells were spun down by centrifuged at 3000 x g for 5 min. The pellet was resuspended in 50 µl water and 10 µl of lyticase solution (5units/ µl in TE) was added to each tube. The cells were resuspended by vortexing and incubated at 37C for 30 min with shaking. 200 µl of buffer P1 (50 mM Tris

pH 8.0; 10 mM EDTA; 100 µg/ml RNase A) was added and vortexed vigorously to mix. 250 µl of buffer P2 (200 mM NaOH; 1%SDS) was then added and mixed gently by inverting 4 -6 times. The samples were then incubated on ice for 5 min. 350 µl of buffer P3 (3M potassium acetate pH 5.0) was added and mixed immediately by inverting 4-6 times, incubated for 15 min on ice. The samples were centrifuged at 14,000 rpm for 10 min and the supernatants were transferred to a fresh tube. 500 µl of isoproponal was added and centrifuged at 14,000 rpm for 10 min at 4C. The pellet was washed with 500 µl of 70% ethanol and centrifuged at 14,000 rpm for 5 min. The resulting pellet was dried and resuspended in 20 µl of DH_2O. The plasmids were then electroporated into *E.coli* and sequenced.

2.2.3 Dictyostelium discoideum

Dictyostelium discoideum a cellular slime mold is a eukaryotic model organism for overexpression of recombinant myosin and myosin fused proteins. This motile amoeba lacks a cell wall and resembles higher eukaryotic cells in behavior and appearance. They carry out mammalian like post-translational modifications and can be readily cultured in petri dishes, shaken flasks, and fermenters. However the abundance of proteolytic enzymes in *Dictyostelium* poses a significant obstacle to the quantitative analysis of levels at which recombinant proteins are expressed in transformed cells. But in the special case of functional myosin motor-fragments, additional protection of the overexpressed protein from post–lysis proteolytic degradation can be achieved by the formation of a tight, insoluble complex with actin. The formation of rigor-like actomyosin complex facilitates also the rapid enrichment and purification of recombinant myosins both on the analytical and preparative scale. The complex can be precipitated and washed to remove the bulk of the cell lysate. Subsequently, functional myosin motors can be selectively released into supernatant by extraction with Mg^{2+}-ATP.

2.2.3.1 Transformation of *D.discoideum* cells

Cells are harvested at a density of 2 to 3 X 10^6 cells/ml by centrifugation at 1,000g for 5 min, washed twice with cold DD-electroporation buffer and resuspended in the same buffer at 1X 10^7 cells/ml (ORF+ cells should be resuspended at 5 to 8 X 10^6 cells/ml). DNA (~ 10-20µg of plasmid) and 0.8 ml of cell suspension are mixed in a microfuge

tube. The mixture is transferred into a 0.4cm electroporation prechilled cuvette and kept on ice for 5 min. the cuvette is flicked to Resuspend settled cells. The outside is dried with a paper wipe and electroporation is carried out immediately with the following settings: 1.2KV, 3μF, 600Ω. The resulting time constant is approximately 0.7 ms. The cuvette is immediately returned to ice. After 5 to 10 min the cells are transferred to a petridish and 10 to 12 ml HL-5C or are added. ORF+ cells should be transferred to 3 to 4 plates per transformation. Cells are allowed to recover for 12 to 34 hrs before applying selection for transformants.

2.2.3.2 Cultivation of *D.discoideum* cells

D.discoideum cells can be grown on tissue culture petri dishes or in a shaking culture at a constant temperature of 20^{o}C. The cells are cultured in HL5c medium containing 10U/ml of penicillin/ streptomycin to prevent bacterial growth and the appropriate concentration of G418 in order to maintain the selection pressure on transformed cells. Untransformed wild type ORF+ cells are grown without G418. Transformed cells harboring a pDX vector with a *neo*^{R} cassette are grown in the presence of 10 to 20μg/ml G418. The concentration depends on the particular construct expressed. Null-cells expressing the HAX1 protein grow in the presence of 10μg/ml. For large scale production, the cells are grown in Erlen-Meyer flasks in shaking culture. The flasks are filled to no more than half their nominal volume with medium and shaken at 180 rpm. Wild type cells and transformants used in this work grow to a density of 1X 10^{7} cells/ml with a doubling time of roughly 12-16 hr. cells are diluted when or before they reach 1X 10^{7} cells/ml, an exchange of the medium is not necessary. Upon dilution, the cell density should not drop below 5X 10^{5}cells/ml. On plates cells are grown until they are confluent and then washed off with medium. Alternatively, cells are washed with Bonner's while adherent on the plate and then washed off with Bonner's for downstream processing. Fresh medium is added and the remaining cells continue growing. Plates can be stored at 4^{o}C for several weeks without exchanging the medium.

2.2.3.3 Conservation of *D.discoideum* spores

Cells are collected from liquid plate or suspension culture. Cells grown to a density of less than or equal to 5 X 10^{6} cells /ml work best. They are washed in MES buffer and Resuspend at 2X 10^{8} cells/ml. 300 ml of this suspension are spread on a MES agar plate.

Plates are dried until there is only a thin film of liquid left (the amount of liquid has a big effect on spore formation). The plates are incubated upside down for 48hr before harvesting the spores by tapping inverted plate smartly on the benchtop. Spores from 3 plates are Resuspend in 1ml 10% glycerol and 100 ml aliquots are frozen by putting them into -80^{o}C freezer. The spores are stored at -80^{o}C.

2.2.3.4 Cryo-conservation of vegetative cells

Vegetative *D.discoideum* cells can be stored frozen without the need to make spores. Cells are washed with cold Bonner's standard solution and Resuspended at high density (>10^{8} /ml) in HL-5c medium containing 10% DMSO. Aliquots of the suspension are put for 2hr in a -20^{o}C freezer. Slow cooling is essential. Subsequently the cells need to be transferred to -80^{o}C freezer. The cells are stored at -80^{o}C. The thawing process should be quick (e.g. warming the tubes on the hand palm) and are transferred immediately to a Petri dish with fresh medium. Once the cells have attached (after about 30 min), the medium is exchanged to get rid of DMSO.

2.2.4 Protein Biochemistry

Purification of Myosin fused mHAX1

Cells expressing a Histidine octomer fusion protein consisting of the catalytic domain of *Dictyostelium* myosin and Murine HAX1were grown in 5 liter flasks containing 2.5 lts of HL-5C medium. The Flasks were incubated in a gyratory shaker at 200 rpm and 21^{o}C. Cells were harvested at a density of 6 X 10^{6} ml^{-1} by centrifugation for 7 min at 2700 rpm in a Beckmann J-6 centrifuge and washed once in PBS. The wet weight of the resulting pellet was determined. Generally, 25g of cell mass was obtained from a 12 liter shaking culture. The cells were resuspended in 120 ml of Lysis buffer (50mM Tris HCl, pH 8.0, 2mM EDTA, 0.2mM EGTA, 1mM Dithiothreitol (DTT), 5mM Benzamidine, 40 µg/ml TLCK, 20 µg/ml N-tosyl-$_{L}$-Phenlylalaninechloromethyl ketone (TPCK), 200 µM 1,10-Phenanthroline,0.04% NaN_3. Cell Lysis was induced by the addition of 60 ml of lysis buffer containing 1% Triton-X-100, 15 mg/ml RNase and 100 units of alkaline Phosphatase. The lysate was incubated on ice for 1 hr. Upon centrifugation at 230,000g for 1 hr the recombinant protein remained in the pellet. The pellet was washed in 100 ml

of HKM buffer (50 mM HEPES, pH 7.3, 30 mM KaC, 10 mM $MgSO_4$, 7 mM β-mercaptoethanol, 5 mM benzamidine, 40 µg/ml PMSF) and centrifuged for 45 min at 230,000g. The recombinant protein was released into the supernatant by extraction of the resulting pellet in 60 ml of HKM buffer with 10mM ATP. After centrifugation (500,000g 45 min), the supernatant was loaded using a peristaltic pump onto a Ni^{2+}-nitriloacetic acid (Ni^{2+}-NTA) affinity column. The flow rate was adjusted to approximately 1.5 ml/min. After loading was complete, the column was washed in Low salt buffer (50mM HEPES, pH 7.3, 30mM KAc, 3 mM benzamidine), High salt buffer (similar to low salt but with 300 mM KAc), and low salt buffer containing 50mM Imidazole. The recombinant mHAX1 was eluted using a linear gradient of Low salt buffer and Imidazole buffer (0.5m Imidazole, pH 7.3, 3mM Benzamidine), starting with 25% Imidazole and reaching 100% after 30 min. The flow rate was 1.5 ml/min and 3 ml fractions were collected and absorbance at 280nm was monitored. SDS gels were run to check the purity of the eluted sample.

2.2.4.1 Preparation of cell lysates for western blot analysis

Cells were washed once with cold PBS and lysed on ice with lysis buffer (see below) for 15 min. For once well of a six well plate 200 µl of lysis buffer was used. Lysates were cleared from cell debris by centrifugation at 3,600 rpm for 9 min at 4 ^oC. For the analysis of proteins of cell lines or transiently transfected cells, the following lysis buffers containing protease inhibitors were used for different applications

1% NP40 Lysis Buffer (a)

20 mM Tris-HCl (pH 7.5)
150 mM NaCL
10 mM $MgCl_2$
2 mM EDTA
10 % Glycerol
1 % NP40
0.1 mM Na_3VO_4
1 mM NaF

1% NP40 Lysis Buffer (b)

20 mM Tris-HCl (pH 7.5)
150 mM NaCL
10 mM $MgCl_2$
2 mM EDTA
10 % Glycerol
1.0 % NP40
2.5 mM β-Glycerophosphate
1 mM NaF

1% Triton X 100 Lysis Buffer

20 mM Tris-HCl (pH 7.5)
150 mM NaCl
1 mM EDTA
1 mM EGTA
1 % Triton X 100
2.5 mM $Na_4P_2O_7$ 10 H_2O
1 mM β-Glycerophosphate
1 mM Na_3VO_4

2% Chaps Lysis Buffer

20 mM Tris-HCl (pH 7.5)
137 mM NaCl
2 mM EDTA
10 % Glycerol
2 % Chaps

Hypotonic buffer

20 mM HEPES (pH 7.6),
10 mM KCl,
1 mM MgCl2,
20% Glycerin,
0.1% Triton X-100,
0.5 mM DTT,
1 mM Na-Orthovanadate,

Hypertonic buffer

20 mM HEPES (pH 7.9)
400 mM NaCl,
1 mM EDTA,
20% Glycerin,
0.1% Triton X-100,
1 mM DTT
1 mM Na-Orthovanadate,

2.2.4.2 Protease inhibitor cocktail

Protease inhibitor cocktail from Sigma Aldrich (P8340) is a mixture of protease inhibitors in DMSO Containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and Aprotinin. Contains no metal chelators.

2.2.4.3 Determination of protein concentration according to Bradford.

To determine the protein concentration according to Bradford (1976) a calibration curve needs to be made by mixing the Bradford reagent with Known concentrations of BSA (10mg/ml = 6.6 OD_{280}) and the protein solution of unknown concentration. After 15 min the absorbance is measured at 595 nm. The protein concentration can be determined with the BSA calibration curve.

To establish the standard curve the following set of standards were used in duplicates.

	0µg/ml	2.5 µg/ml	5 µg/ml	10 µg/ml
BSA 1mg/ml	0 µl	2.5 µl	5 µl	10 µl
Filtered Bradford	200 µl	200 µl	200 µl	200 µl
dH_20	800 µl	797.5 µl	795 µl	790 µl

Samples: 5 µl dilution of 1:10 or 5 µl of undiluted sample in 795 µl of dH_20 + 200 µl of filtered Bradford was used.

2.2.4.4 Preparation of cell lysates for Immunoprecipitation

Cells were washed once with cold PBS and 2% Chaps Lysis buffer (1ml per 10cm plate) with complete protease inhibitor cocktail (Sigma Aldrich) was added (adherent) to the cells on the plate, incubated for 5 min and then the cells were collected using a cell scrapper to a falcon tube. The cells were incubated on ice for 10 min and then the cells were lysed using a syringe and needle (23G 1¼). The lysates were incubated for an additional 10 min on ice. The lysates were cleared from cell debris by centrifugation at 3,600 rpm for 9 min at 4°C.

2.2.4.5 Preparation of *D.discoideum* protein lysate

The medium from a confluent 9 cm petri dish of *D.discoideum* is removed and the cells are washed on the plate with Bonner's solution. The cells are then harvested in 1 ml of Bonner's, transferred to a 1.5ml eppendorf tube and centrifuged at 3000 rpm in a bench top centrifuge. The supernatant is removed and the cells are lysed for 10 min at room temperature by addition of 1 ml of LB_{50} containing protease inhibitors and 0.5% Triton X 100.The tubes are centrifuged for 30 min at full speed at 4°C. The supernatant is discarded. An aliquot can be saved for SDS-PAGE or western blot. Protein is solubilized by homogenizing the pellet with 50 µl of LB300 containing 10 mM ATP using an Eppendorf plastic pistil. After 20 min of centrifugation at full speed the supernatant and the pellet are analyzed by SDS-PAGE.

2.2.4.6 SDS polyacrylamide gel electrophoresis

Proteins can be separated by their size on SDS polyacrylamide gels. Depending on the size of the protein, gels with 10% to 15% polyacrylamide are used. Minigels with a size of about 6cm X 8cm and 10 to 15 wells are used. The protein sample is diluted with 3X protein loading buffer, boiled at 95 ^{o}C for 9 min and 5-20 µl of sample were loaded per lane depending upon the experiment. Gels were run at 180V for 60 min approximately in a Hoefer mighty small electrophoresis chamber (BIORAD).

Protein loading buffer (5X)

300 mM Tris-HCl, pH 6.8
50 % (v/v) Glycerol
10 % SDS (w/v)
0.1 % (w/v) Bromophenol blue
300 mM β-Mercaptoethanol.

Coomassie stain

Coomassie brilliant blue R250 2g
Coomassie brilliant blue G250 0.5g
Methanol 50ml, Ethanol 450ml,
Glacial acetic acid 100ml, DH20 400ml

Electrophoresis buffer:

25 mM Tris-Base
250 mM Glycine
0.1% (w/v) SDS
pH 8.3

Destain

Ethanol 5.0%
Acetic acid 7.5%

For detection of proteins after SDS-PAGE, gels were blotted or stained. For staining, gels were washed once in H_2O, stained for 1 hr in coomassie blue staining solution and destained in destain solution for 1 hr.

2.2.4.7 Immunoblotting

Blotting or Transfer Buffer

50 mM Tris (base), 380 mM Glycine, 0.1 % SDS, 20% Methanol
pH does not need to be adjusted.

PonceauS

PonceauS 0.2%
Trichloroacetic acid 3%

The proteins can be stained on the membrane with PonceauS S solution.

Blocking solution

Skim milk powder 5% in PBST or 5% BSA in TBST.

Proteins are transferred to a membrane by electrophoresis. The bound protein is then specifically detected using an enzyme linked antibody and an enzymatic reaction. Wet transfer of proteins used for transfer. PVDF membrane (Polyvinylidene Fluoride) is first activated by soaking it in methanol for two minutes and then equilibrated in blotting buffer. The gel is also briefly equilibrated in the blotting buffer. The blot is assembled by sandwiching in a plastic support – from bottom to top – a sponge, whatmann paper, the gel, the membrane, whatmann paper and another sponge. All components should be prewetted and there must be no air bubbles between the gel and the membrane. The sandwich is placed on the cathode and applied such that the anode is at the end of the gel. The transfer is done at 4 ^{o}C. A blotting time of 1 Hr at 400mA is usually sufficient for minigels.

For Specific detection, the membrane is incubated with an antibody directed against the protein of interest (primary antibody), washed and then incubated with an enzyme linked secondary antibody directed against the Fc part of antibodies from the exact species in which the primary antibody was raised. First, the membrane is incubated in the blocking solution for 1 hr at room temperature. Then the membrane is incubated in blocking solution containing an appropriate dilution of the primary antibody at 4 ^{o}C overnight on a shaker. The membrane is rinsed briefly with water and washed 3 times for 10 min with PBST. The incubation with secondary antibody is done for 1 hr at room temperature and the membrane is washed again three times with PBST for 10 min each. For detection, The ECL^{TM} western blotting detection reagent (Amersham Pharmacia) or SuperSignal$^{®}$ West Pico chemiluminiscent substrate (Thermo Scientific) was used according to the manufacturers' instructions.

2.2.5 Mammalian cell splitting and transfection:

2.2.5.1 Cell splitting

Media was drained from the cell culture by suction and cells were washed with sterile PBS. Cells were incubated in trypsin for 2min at 37 ^{o}C to dislodge the cells from the surface. Dislodged cells were resuspended in culture media and seeded on a fresh plate.

2.2.5.2 Cell maintenance:

Different cell lines were maintained at Dulbecco's modified Eagles medium supplemented with 10% FCS and 1% Penicillin/streptomycin (V/V) at 37 ^{o}C in a humidified atmosphere containing 5%CO_2.

2.2.5.3 Cell transfection:

Cells to be transfected were seeded in 10 cm cell culture plate up to 60% confluency and incubated at 37 ^{o}C overnight for adherence. 12.5 µg of Plasmid DNA was dissolved in 676 µl of Plain DMEM medium and 44.6 µl of 1 µg/ml PEI was added to it and mixed by vortexing. The solution was allowed to incubate for 10 min at Room Temperature. The medium in the plate was replaced with 6.6 ml of fresh complete medium with out any antibiotics. The transfection mixture was added to the cells and incubated for 12 to 48 hrs for optimal expression of the proteins.

2.2.5.4 Mammalian cell fixation

2.2.5.4.1 PFA fixation

Cells cultured on cover slips were briefly rinsed with warm PBS and incubated in a freshly thawn 4% paraformaldehyde in PBS at RT. Fixed cells were washed twice with PBS and quenched with 50mM NH_4Cl , washed once again with PBS and permeabilized with 0.5% Triton-X-100 for 4 min at RT. Fixed cells were washed with PBS two more times for 5 min and proceeded with immunolabelling protocol.

2.2.5.5 Immunofluorescence microscopy:

After the cells were fixed, they were incubated with primary antibodies diluted in filtered PBS with 0.5% BSA for 1 hr at room temperature. Secondary antibodies used were goat anti rabbit Alexa 488, goat anti mouse Alexa 546, and goat anti rabbit Alexa 633.

2.2.5.6 Confocal Microscopy:

Confocal images of immunolabelled specimens were obtained using the confocal laser scanning microscope TCS-SP (Leica) equipped with a 63X PL Fluotar 1.32 oil immersion objective. An argon laser and Helium-neon lasers for exitation of fluorescence were used. For simultaneous multichannel acquisition, emission signal of different probes was acquired using the appropriate wavelength settings for each photomultiplier. The images from different channels were independently attributed with color codes and then superimposed using the accompanying Leica software and Adobe Photoshop.

2.2.5.6.1 Colocalization Analysis:

Analysis of the images for colocalization, taken by confocal laser scanning microscope was done using WCIF Image J software for Pearson's correlation coefficient, Mander's coefficient and percentage of overlap. **Rr** - represents Pearson's correlation coefficient. This correlation analysis the values for Pearson's will range from 1 to -1. Value of 1 represents perfect correlation; -1 represents perfect exclusion and zero represents random localization. **R -** represents Mander's Overlap coefficient. It ranges from 1 to zero with 1 being very high co-localization. **ch1:ch2** value represents the red: green pixel ratio. **M1** and **M2** are Mander's Colocalization coefficients for channel 1 (M1) and channel 2 (M2).

2.2.5.7 Cell proliferation and Viability by MTT:

The MTT assay, widely used to measure cell proliferation is based on reduction of the tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by actively growing cells to produce a blue formazan product. Cells were cultured in a 96 well flat bottomed tissue culture plate. 10 µl of the MTT labeling reagent was added to each sample and incubated for 4hrs at 37C, then 100 µl of solubilization reagent was added and incubated overnight at 37C. The microplate was subjected to Elisa reader for Absorbance at 550nm and 690 nm. The corrected value $A550_{nm}$-$A690_{nm}$ was used for analysis.

2.2.5.8 Generation of Retroviruses

Recombinant VSV-G pseudotyped retroviruses were generated using transient transfection into the packaging cell line 293 T. 293T cells were transfected using a

standard calcium phosphate transfection protocol. 45 µg of Plasmid DNA, 147 µl of 2M $CaCl_2$ and Low TE up to 1.2 ml were mixed and then added to 2X HBS by dropping down the side of the tube while bubbling air into HBS solution using an electric pipette. The mixture was left to stand at room temperature for 30 minutes. The fine white cloudy precipitate was dropped onto the surface of the 293T cells. After 8-12 hours the medium was replaced with fresh complete DMEM medium. Supernatants were collected at time points 24Hrs, 36 hrs and 48 hrs post transfection respectively. The filtered supernatants were stored at-80^{o}C till further use.

2.2.5.9 Determination of Viral Titer

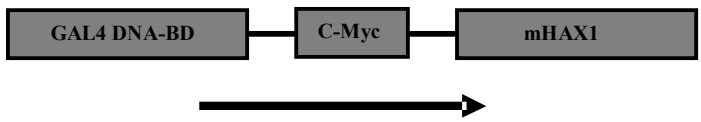
The viral titers were determined by infecting A549 cells with serial dilutions of thawed vector stock, in the presence of 8 µg/ml of polybrene in complete medium. Infected cells were harvested after three days, resuspended in PBS and analyzed by FACS to determine the percentage of surrogate marker-expressing cells. Viral titers were calculated multiplying the initial number of cells by the dilution factor of the viral supernatant by the percentage of surrogate marker -expressing cells and divided by the initial volume of medium. The final titer was determined as the average of the titers obtained in each dilution

3. Results

3.1 The first goal of this thesis is to screen for proteins which interact with HAX1 using Yeast Two Hybridization technique.

3.1.1 Cloning

In this regard Full length Murine Hax1 was cloned into pGBKT7 vector which expresses proteins fused to the amino acids 1-147 of the GAL4 DNA binding domain (DNA-BD). This plasmid encoding mHAX1 cDNA was constructed by amplifying the cDNA of mouse lymph node by PCR. The amplified PCR fragment was then subcloned into pGEM-T plasmid and confirmed by sequencing. The mHAX1 cDNA was released by digesting it with BspH1 and Sal1 and cloned into Nco1 and Sal1 sites of pGBKT7.



3.1.2 Yeast Transformation and Transactivation

pGBKT7-mHAX1 was transformed into Yeast AH109 cells by Lithium acetate method and the cells were plated on **SD Agar ΔHT**, **SD Agar ΔAT** and **SD Agar ΔAHT** to check for transactivation of the bait protein. Colonies were observed only on **SD Agar ΔHT** plate and there were no colonies on **SD Agar ΔAT** and **SD Agar ΔAHT** plates**.** This confirmed that there was no Transactivation by **mHAX1-pGBKT7.**

3.1.3 Protein expression

Colonies from **SD Agar ΔHT** plate were picked and cultured in **SD ΔT medium**. The cells were harvested in YEX lysis buffer and the extracts were loaded on 10% SDS page gel and blotted for HAX1 to confirm its expression.

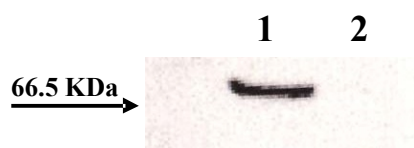


Fig 10: Confirmation of mHAX1-pGBKT7 Protein expression: Yeast AH109 lysates of mHAX1-pGBKT7 and pGBKT7 only were loaded on 10% SDS page gel and detected for HAX1 using HAX1 antibody. Lane 1: mHAX1-pGBKT7, Lane2: pGBKT7.

3.1.4 Transformation with 17 days mouse embryonic cDNA library:

AH109 cells expressing mHAX1 was mated together with the 17day mouse embryonic cDNA library clones in Yeast Y187 strain and the cultures were plates on **SD Δ AHLT agar** plates. Colonies were observed on these plates after 5 days of incubation at 30^{o}C.

3.1.5 X-Gal colony Lift filter assay

The colonies were replica platted on **SD Δ AHLT** agar plates and subjected to β-galactosidase X-Gal colony lift filter assay on Whatmann filters. The filters were incubated in dark at RT for 3 hrs. Blue colonies observed were labeled and cultured for Plasmid isolation.

3.1.6 Isolation of plasmids from Blue colonies:

The positive blue colonies were cultured in **SD Δ Leu** medium, their plasmids were extracted and electroporated into *E.coli* and sequenced. The sequenced results were analyzed using BLAST programme.

The Table below lists the probable interacting proteins of mHAX1 fished by the Yeast Two Hybrid Screen.

Table 1:

No	**POTENTIAL INTERACTING PARTNERS**	**ACCESSION NO**
1	Mus musculus surfactant associated protein C (Sftpc), mrna	NM_011359.1
2	Mus musculus proteosome (prosome, macropain) subunit, beta type 1 (psmbl), mrna	NM_011185
3	Histone cell cycle regulation defective interacting protein 5	AK003730
4	Mus musculus procollagen, type III, alpha 1, mrna	BC058724
5	Cytochrome C oxidase subunit 2	AY999076
6	Mus musculus adaptor protein complex AP-2, mu1, MRNA	BC056352
7	NADH dehydrogenase subunit 5	AJ512208
8	TNF receptor – associated factor 3 interacting protein 1 (Traf3ip1), mrna	NM_028718 XM_129927
9	Mus musculus serine (or cysteine) peptidase inhibitor, clade B, member 1a, mrna.	BC104333

10	Mus musculus mu-protocadherin mrna	AF462391
11	Casein kinase II, alpha 2, polypeptide.	NM_009974
12	Bone morphogenetic protein 1 (BMP1), mrna	NM_009755
13	Hypothetical protein, Mus musculus coiled-coil domain containing 56 (Ccdc56), mRNA	AKO19025
14	Mus musculus integral membrane protein 2A, mrna	NM_008409
15	Putative tumor differentially expressed 2	AK141574
16	SMAD specific E3 ubiquitin protein ligase 2, transcript variant 2 (smurf2), mrna	XM_001001809
17	Mus musculus Tp53rk binding protein (TprKb), mrna	NM_176842
18	SMC 4 structural maintenance of chromosomes 4 - like 1	NM_133786
19	Mus musculus transmembrane protein 45b (Tmem45b), mrna	NM_144936
20	Similar to prochymosin isoform 2	XM_987135
21	ATPase, H+ transporting, lysosomal (vacuolar proton pump) Unnamed protein product.	AK088712
22	Eukaryotic translation initiation factor 3, subunit 8 (Eif3s8) pseudogene Similar to stathmin 1 stmn1	AL845317
23	Similar to SH3 domain GRB2- like protein B1 (Endophilin B1)	XM_988661
24	Mus musculus amylase 2a2, pancreatic (Amy2a2), mRNA	NM_001160152

Clone 1 Mus musculus surfactant associated protein C (Sftpc), mRNA

Accession no NM_011359.1, This clone was fished once.

This has sequence similarity from 42 bp to 617 bp

GACATGAGTAGCAAAGAGGTCCTGATGGAGAGTCCACCGGATTACTCGGCAG
GTCCCAGGAGCCAGTTCCGCATCCCCTGCTGTCCCGTGCACCTCAAACGCCTT
CTCATCGTGGTTGTGGTGGTGGTCCTCGTTGTCGTGGTGATTGTAGGGGCTCT
GCTCATGGGCCTCCACATGAGTCAAAAACATACTGAGATGGTCCTTGAGATG
AGCATCGGAGCACCGGAAACTCAGAAACGCCTAGCCCCGAGTGAGCGAGCA
GACACCATCGCTACCTTTTCCATCGGCTCCACTGGCATCGTTGTGTATGACTA
CCAGCGGCTCCTGACGGCCTATAAGCCAGCTCCAGGAACCTACTGCTACATC
ATGAAGATGGCTCCAGAGAGCATCCCTAGTCTTGAGGCTTTCGCTAGAAAA
CTCCAGAACTTCAGGGCCAAGCCCTCCACACCCACCTCTAAGCTGGGCCAGG
AGGAAGGGCATGATACTGGTTCCGAGTCCGATTCTTCCGGGAGAGACCTGGC

TTTCCTAGGCCTTGCTGTGAGCACCCTGTGTGGAGAGCTACCACTCTACTATA
TC

Open reading frame- protein sequence

D **M** S S K E V L **M** E S P P D Y S A G P R S Q F R I P C C P V H L K R L L I V V V
V V V L V V V V V I V G A L L **M** G L H **M** S Q K H T E **M** V L E **M** S I G A P E T Q
K R L A P S E R A D T I A T F S I G S T G I V V Y D Y Q R L L T A Y K P A P G T
Y C Y I **M** K **M** A P E S I P S L E A F A R K L Q N F R A K P S T P T S K L G Q E E
G H D T G S E S D S S G R D L A F L G L A V S T L C G E L P L Y Y I

Clone 2:

Mus musculus proteosome (prosome, macropain) subunit, beta type 1 (psmbl), mRNA.

Accession: NM_011185, This clone was fished 2 times

This has sequence similarity from 4bp to 695 bp.

AGTGGCCATTTTGCCGTGAGACAGCAGGAGTTAGGCGCGATGCTTTCCACTG
CTGCTTACCGAGACGTGGAACGAGAGCTGGGAATGGGGCCTCACGGCTCCGC
CGGCCCTGTGCAGCTGCGGTTTTCGCCTTATGCCTTCAACGGAGGTACTGTAT
TGGCAATTGCTGGAGAAGATTTTTCCATCGTCGCTTCAGACACTCGATTGAGT
GAAGGATTTTCAATTCATACCCGAGATAGCCCCAAATGCTACAAACTGACAG
ACAAGACAGTAATTGGCTGCAGTGGTTTCCATGGAGATTGTCTCACTTTGACA
AAGATTATTGAAGCAAGATTAAAGATGTACAAGCATTCCAATAACAAGGCCA
TGACAACGGGGGCGATTGCTGCAATGCTGTCTACCATCCTGTACTCACGGCG
CTTCTTCCCTTACTATGTTTACAACATCATTGGAGGACTTGATGAAGAAGGAA
AGGGAGCTGTGTACAGCTTTGACCCAGTGGGCTCTTACCAGAGAGACTCTTTC
AAGGCGGGAGGCTCAGCAAGTGCCATGCTGCAGCCTCTGCTCGACAACCAGG
TTGGCTTMAAAATATGCAGAATGTGGAGCACGTCCCCCTGACGCTGGACAGA
GCCATGAGGCTGGTGAAAGATGTCTTCATTTCTGCAGCCGAGAGGGATGTGT
ATACTGGAGATGCTCT

Open reading frame-protein sequence

S G H F A V R Q Q E L G A **M** L S T A A Y R D V E R E L G **M** G P H G S A G P V
Q L R F S P Y A F N G G T V L A I A G E D F S I V A S D T R L S E G F S I H T R D
S P K C Y K L T D K T V I G C S G F H G D C L T L T K I I E A R L K **M** Y K H S N

N K A **M** T T G A I A A **M** L S T I L Y S R R F F P Y Y V Y N I I G G L D E E G K G A V Y S F D P V G S Y Q R D S F K A G G S A S A **M** L Q P L L D N Q V G X K I C R **M** W S T S P **Stop** R W T E P **Stop** G W **Stop** K **M** S S F L Q P R G **M** C I L E **Met** L

Clone 3:

Mus musculus Histone cell cycle regulation defective interacting protein 5.

Accession: AK003730, This clone was fished once.

This has sequence similarity from 1 bp to 712 bp

TGGCCATTATGGCCGGGGGGCACTGAAGTGATGGCGGCGGCCGAGCGGGCAT
GGGGAGCAGCGGTCGGTGTAGTCCGGCTGTGCAGGCGGTTCTGTCATGTAGC
GACTCCACACACCTTTAAGAAACAACCTCTGCATCAATATGCACGGAGACCA
CTTTTCCCGCTGCGCACACCCTTGTGTAACACAGTACGATTCATGTTTATTCA
AACACAAGACACCCCAAATCCCAACAGCTTAAAGTTTATACCAGGAAAGCCG
GTTCTTGAGACGAGGACCATGGATTTCCCCACCCCTGCTGCAGCTTTCCGCTC
CCCTCTGGCCAGGCAGTTATTCAGAATTGAAGGTGTGAAAAGCGTCTTCTTCG
GACCAGATTTCATCACAGTCACAAAGGAGAATGAAGAGTTAGACTGGAATTT
ACTGAAACCAGACATTTATGCCACAATTATGGACTTCTTTGCATCTGGCTTAC
CCCTAGTGACCGAGGAGACGCCTCCGCCTCCGGGAGAAGCAGGCTCTGAGGA
AGATGACGAAGTCGTGGCAATGATTAAAGAACTGCTAGATACTAGAATAAGG
CCAACTGTGCAGGAGGATGGGGGCGACGTGATCTACAGGGGCTTTGAAGATG
GCATCGTGCGGCTGAAGCTCCAGGGCTCCTGCACCAGCTGCCCCAGCTCCMT
CATCACCCTGAAGAGCGGGATTCAGAACATGCTGCAGTTTTATATTCCA

open reading frame-protein sequence

W P L W P G G T E V **M** A A A E R A W G A A V G V V R L C R R F C H V A T P H T F K K Q P L H Q Y A R R P L F P L R T P L C N T V R F **M** F I Q T Q D T P N P N S L K F I P G K P V L E T R T **M** D F P T P A A A F R S P L A R Q L F R I E G V K S V F F G P D F I T V T K E N E E L D W N L L K P D I Y A T I **M** D F F A S G L P L V T E E T P P P P G E A G S E E D D E V V A **M** I K E L L D T R I R P T V Q E D G G D V I Y R G F E D G I V R L K L Q G S C T S C P S S X I T L K S G I Q N **M** L Q F Y I P

Clone 4

Mus musculus procollagen, type III, alpha 1, mRNA

Accession: BC058724, This clone was fished once.

This has sequence similarity from 2882 bp to 3037 bp and again from 3165 bp to 3451 bp

TGGCCATTATGGGGGACGTGGTCCTCCTGGACCCCAAGGTCTTCCTGRTMAR
CCTGGTACAGMTGSTGAMCCTGGAAGGGATGGAAACCCTGGATCAGATGGT
CAGCCAGGTCGAGATGGATCTCCTGGTGGCAAGGGTGATCGTGRTGAAAATG
GTTCTCCTGGTGCCCCAGGCGCTCCTGGTCATCCAGGCCCACCTGGCCCTGTT
GGTCCATCTGGAAAGAGTGGTGACAGAGGASAAACTGGCCCTGCTKGTCCTT
CTGGWGCCCCAGGACCCAGTGGTGCTCCTGGCAAGGACGGMCCTCCAGGTC
CTGCAGGCAACAGTGGTTMTCCTGGCAMCCCTGGAATAGMTGGACCAAAAG
GTGATGCTGGACASCCTGGAGAGAAGGGGCCACCTGGTGCTCARGGTCCTCC
GGGTTCCCMARG

Open reading frame- protein sequence

G H Y G G R G P P G P Q G L P X X P G T X X X P G R D G N P G S D G Q P G R D
G S P G G K G D R X E N G S P G A P G A P G H P G P P G P V G P S G K S G D R
G X T G P A X P S X A P G P S G A P G K D X P P G P A G N S G X P G X P G I X G
P K G D A G X P G E K G P P G A X G P P G S X X

Clone 5 cytochrome c oxidase subunit II Mus musculus

Accession no AY999076, This clone was fished 16 times.

This has sequence similarity from 3bp to 714 bp

GTGGCCATTATGGCCTACCCATTCCAACTTGGTCTACAAGACGCCACATCCCC
TATTATAGAAGAGCTAATAAATTTCCATGATCACACACTAATAATTGTTTTCC
TAATTGGCTCCTTAGTCCTCTATATCATCTCGCTAATATTAACAACAAAACTA
ACACATACAAGCACAATAGATGCACAAGAAGTTGAAACCATTTGAACTATTC
TACCAGCTGTAATCCTTATCATAATTGCTCTCCCCTCTCTACGCATTCTATATA
TAATAGACGAAATCAACAACCCCGTATTAACCGTTAAAACCATAGGGCACCA
ATGATACTGAAGCTACGAATATACTGACTATGAAGACCTATGCTTTGATTCAT
ATATAATCCCAACAAACGACCTAAAACCTGGTGAACTACGACTGCTAGAAGT

TGATAACCGAGTCGTTCTGCCAATAGAACTTCCAATCCGTATATTAATTTCAT
CTGAAGACGTCCTCCACTCATGAGCAGTCCCCTCCCTAGGACTTAAAACTGAT
GCCGTCCCAGGCCGACTAAATCAAGCAACAGTAACATCAAACCGACCAGGGT
TATTCTATGGCCAATGCTCTGAAATTTGTGGATCTAACCATAGCTTTATGCCC
ATTGTCCTAGAAATGGTTCCACTAAAATATTTCGAAAACTGATCTGCTTCAAT
AATTTAATCTANAAAAAAAAAAAAAAAAAAAAACATGTCGGCCGCCTCGG
CCTCTAG

Open reading frame-protein sequence

V A I **Met** A Y P F Q L G L Q D A T S P I I E E L I N F H D H T L I I V F L I G S L V
L Y I I S L I L T T K L T H T S T I D A Q E V E T I **Stop** T I L P A V I L I I I A L P S
L R I L Y I I D E I N N P V L T V K T I G H Q **Stop** Y **Stop** S Y E Y T D Y E D L C F
D S Y I I P T N D L K P G E L R L L E V D N R V V L P I E L P I R I L I S S E D V L
H S **Stop** A V P S L G L K T D A V P G R L N Q A T V T S N R P G L F Y G Q C S E
I C G S N H S F **Met** P I V L E **Met** V P L K Y F E N **Stop** S A S I I **Stop** S X K K K K
K K K H V G R L G L **Stop**

Clone 6 Mus musculus adaptor protein complex AP-2, mu1 (Ap2m1), mRNA

Accession: BC056352, This clone was fished twice.

This has sequence similarity to 23 to 721 bp of AP-2, mu1 gene.

TGGCCATTATGGCCGGGGAGGCGAGCTGGGGGCGGGCGGACATCTTGGGATC
CGARAGTGGCCAGGCCSGCCTTGTGGGGGCTGCTGACTCCAGGTCTGTTCTCA
RAGCAATGGGTCACAGAGACTGAGCTGCCACCATGATCGGAGGCTTATTCAT
CTATAATCACAMGGGGGAGGTGCTTATCTCCCGGGTCTACAGAGATGACATC
GGGAGGAATGCTGTGGATGCCTTTCGGGTCAATGTYWTTYWTGCACKGCAG
CAGGTGCGCAGCCCTGTCACAAACATCGCTCGCACCAKCTTCTTCCATGTTAA
GCGGKCCAACATCTGGCTGGCCGYASTCACCAAGCAGAATGTCAATGCTGCC
ATGGTCTTCKAATTCCTCTACAAGATGTGTGATGTAATGGCTGCTTACTTTGG
CAAAATCAGCGAGGAGAACATSAAGAACAATTTTGTGCTCATATACGAGCTG
CTGGATGAGATTCTGGACTTTGGCTACCCACAGAACTCAGAGACAGGTGCAC
TGAAAACCTTCATCACCCGGCAGGGTATCAAGAGTCAGCATCAGACGAACGA

AGAACAGTCCCAGATCACCRGCCAGGTGACCGGGCARATTGGCTGGCGGTGA
GAASGCATCAMGTATCGCCGGAATGAACTCTTCCTAGATGTTCTGGAGAGTG
TGAACCTGCTTATGTCCCCACAGGGGCAGTGCTGAGTG

Open reading frame- protein sequence

A I **M** A G E A S W G R A D I L G S X S G Q A X L V G A A D S R S V L X A **M** G H R D **Stop** A A T **M** I G G L F I Y N H X G E V L I S R V Y R D D I G R N A V D A F R V N X X X A X Q Q V R S P V T N I A R T X F F H V K R X N I W L A X X T K Q N V N A A **M** V F X F L Y K **M** C D V **M** A A Y F G K I S E E N X K N N F V L I Y E L L D E I L D F G Y P Q N S E T G A L K T F I T R Q G I K S Q H Q T N E E Q S Q I T X Q V T G X I G W R **Stop** E X I X Y R R N E L F L D V L E S V N L L **M** S P Q G Q C **Stop** V

Clone 7 NADH dehydrogenase subunit 5

Accession no NC_005089.1, This clone was fished twice.

This clone has sequence similarity to 7013 to 7460 bp

TGGCCATTATGGCCTACCCATTCCAACTTGGTCTACAAGACGCCACATCCCCT
ATTATAGAAGAGCTAATAAATTTCCATGATCACACACTAATAATTGTTTTCCT
AATTAGCTCCTTAGTCCTCTATATCATCTCGCTAATATTAACAACAAAACTAA
CACATACAAGCACAATAGATGCACAAGAAGTTGAAACCATTTGAACTATTCT
ACCAGCTGTAATCCTTATCATAATTGCTCTCCCCTCTCTACGCATTCTATATAT
AATAGACGAAATCAACAACCCCGTATTAACCGTTAAAACCATAGGGCACCAA
TGATACTGAAGCTACGAATATACTGACTATGAAGACCTATGCTTTGATTCATA
TATAATCCCAACAACGACCTAAACCTGGTGAACTACGACTGCTAGAAGTGAT
ACCGAGTCGTTCTGCCATAGAACTTCCA

Open reading frame-protein sequence

W P L W P T H S N L V Y K T P H P L L **Stop** K S **Stop Stop** I S **Met** I T H **Stop Stop** L F S **Stop** L A P **Stop** S S I S S R **Stop** Y **Stop** Q Q N **Stop** H I Q A Q **Stop Met** H K K L K P F E L F Y Q L **Stop** S L S **Stop** L L S P L Y A F Y I **Stop Stop** T K S T T P Y

Stop P L K P **Stop** G T N D T E A T N I L T **Met** K T Y A L I H I **Stop** S Q Q R P K P G E L R L L E V I P S R S A I E L P

Clone 8 Mus musculus TRAF3 interacting protein 1, mRNA

Accession no NM_028718, XM_129927, This clone was fished once.

This has sequence similarity from 895bp to 1612bp

TGGAGGCNGTGAATTCNCCCAAGCAGTGGTATCAACGCAGAGTGGCCATTAT
GGCCGGGGACCGGGAACGGCGGAAATCGAAGAATGGAGAACATACCCGGGA
TCCTGACAGGGAGAAAAGCAGAGATGCAGACAAGCCAGAGAAAAAGTCCTC
AAGCTCAGGGGAGATATCTAAAAAGCTGTCAGATGGATCTTTCAAAGATGTC
AAAGCAGAGATGGAGGCTGACATTTCTGTGGGAGCATCTAGGTCCTCGACGT
TAAAACCATCAAAACGGCGATCGAAGCATTCACTGGAAGGTGACTCCCCCAG
CGATGCAGAAGTAGAAGCTGGACCTGCTGGCCAAGATAAGCCGGAGGTGAT
GGAGAATGCGGAAGTTCCCAGTGAGCTTCCCTCCAGCCTCAGAAGAATACCT
CGGCCTGGGAGTGCACGGCCAGCACCTCCCAGGGTCAAACGACAAGAGAGC
ACAGAGACACTGGTGGTAGACAGGTCGGGAAGTGGTAAAACCGTCTCCAGTG
TGATCATCGACTCCCAGAACTCTGACAATGAGGATGACGAGCAGTTTGTGGT
GGAGGCTGCCCCTCAGCTGTCAGAAATCGCAGACATTGACATGGTGCCGTCA
GGGGAGCTGGAGGATGAGGAGAAGCACGGTGGGCTTGTGAAGAAGATCTTG
GAGACGAAGAAGGATTATGAGAAATTGCAGCAGTCTCTCAAGCCTGGTGAGA
AGGAGAGATCACTCATCTTTGAGTCAGCATGGAAGAAGGAGAAAGACATCGT
TT

Open reading frame-protein sequence

E X V N X P K Q W Y Q R R V A I **Met** A G D R E R R K S K N G E H T R D P D R E K S R D A D K P E K K S S S S G E I S K K L S D G S F K D V K A E **Met** E A D I S V G A S R S S T L K P S K R R S K H S L E G D S P S D A E V E A G P A G Q D K P E V **Met** E N A E V P S E L P S S L R R I P R P G S A R P A P P R V K R Q E S T E T L V V D R S G S G K T V S S V I I D S Q N S D N E D D E Q F V V E A A P Q L S E I A D I D **Met** V P S G E L E D E E K H G G L V K K I L E T K K D Y E K L Q Q S L K P G E K E R S L I F E S A W K K E K D I V

Clone 9 Mus musculus serine (or cysteine) peptidase inhibitor, clade B, member 1a, mRNA.

Accession no BC104333, This clone was fished twice.

This has sequence similarity from 112 bp to 733 bp

GTGAATTCMCCCAAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGGG
ATCCTAGCTGTAAGTGGAGCCAGACCTGCTAAGCAAGAGACTTCACCATGGA
GCAGCTGAGTTCAGCCAACACCCTCTTCGCCTTGGAGCTGTTCCAAACCCTGA
ATGAAAGCAGCCCCACAGGAAACATCTTCTTCTCTCCCTTCAGCATTTCTTCT
GCCTTGGCCATGGTCATTCTGGGGGCCAAAGGCAGCACTGCAGCTCAGCTTT
CTAAGACTTTTCATTTTGACTCTGTTGAGGACATCCATTCACGCTTCCAAAGC
CTGAATGCTGAAGTGAGCAAACGTGGAGCATCTCACACTCTGAAACTTGCTA
ACGGACTGTATGGAGAGAAAACCTACAACTTCCTTCCTGAATACTTGGCTTCA
ACCCAGAAAATGTATGGTGCTGACCTGGCCCCTGTGGATTTTCTGCATGCCTC
TGAGGATGCAAGGAAGGAGATAAACCAGTGGGTCAAAGGTCAAACAGAAGG
GAAAATCCCAGAACTGTTGTCTGTGGGTGTGGTGGACAGTATGACCAAACTT
GTGCTGGTGAATGCCATCTACTTTAAGGGAATGTGGGAGGAGAAATTCATGA
CAGAGGACACAACGGATGCTCCATTCCGACTGAGTAAGAAAGACACAAAAA
CAGTGAAGATGATGTATCAAAAGAAAAAATTTCCATTTGGTTACATTTCGGA
CCTGAAGTGCAAGGTGCTGGAGATGCCTTACCAGGGTGGAGAACTTAGCATG
G

Open reading frame-protein sequence

V N X P K Q W Y Q R R V A I **M** A G D P S C K W S Q T C **Stop** A R D F T **M** E Q L
S S A N T L F A L E L F Q T L N E S S P T G N I F F S P F S I S S A L A **M** V I L G A
K G S T A A Q L S K T F H F D S V E D I H S R F Q S L N A E V S K R G A S H T L
K L A N G L Y G E K T Y N F L P E Y L A S T Q K **M** Y G A D L A P V D F L H A S
E D A R K E I N Q W V K G Q T E G K I P E L L S V G V V D S **M** T K L V L V N A
I Y F K G **M** W E E K F **M** T E D T T D A P F R L S K K D T K T V K **M M** Y Q K K
K F P F G Y I S D L K C K V L E **M** P Y Q G G E L S **M**

Clone 10 Mus musculus mu-protocadherin mRNA

Accession no AF462391, This clone was fished once.

This has sequence similarity from 936 bp to 1747bp

GGAGGCNGTGAATTCMCCCAAGCAGTGGTATCAACGCAGAGTGGCCATTATG
GCGGGAAACACAGATGGCACATTCATCATCAACGCACACGATGGCAACCTCA
CAATGACCAAAAGTATCCCGAGCCCCATGAAGTTCACCCTTCTGATCAGGGC
TGATCAGGAAGACATGGCTCAATACTCAGTGACCCAGGCCATCGTGGAGGCT
CGCAGTGTCACTGGAAACCCACTCCAGTTCTCCCAGAGCCTGTACTATGGCA
CAGTGGTACTGGGATCTGAGGCTGGCACAGCAGTGAAGGACAAGACCTTCCC
TTCGGAGATCCTGAGGATCCAGGCTCAGTACCCAGGCTTCCCGGACCTCAAC
TCAGCTGTCACATATCGAGTCACCAACTCCTCAGAATTCATGATGAATAAGG
ATATCATGCTGACTGCTGTGCCTATGGAGGAAGCAAGAACCATCCGTGTAGA
GGTAGAAGCTAGCAATACTGTGACTAAGGACACAGCCACCGCTGTTGTTGAG
ATCCAGGTGTCAGAGCGAGAACTGCCCTCCACAGAAGCTGGAGGAACGACT
GGGCCTTCAAGTAACACCACTATGGAAGCCCCCTTGACCTCTGGGACCTCTC
AAAGACCTGCCACCACCAGCTCTGGGGGAAGTGTTGGCCCATTCCCACCTGG
TGGCACAACTCTAAGACCACCCACCCCTGCCTCATCCATACCTGGGGGGTCC
CCTACTCTTGGAACCAGCACCTCTCCCCAGACAACCACTCCTGGTGGGGACTC
AGCACAGACCCCCAAACCAGGAACCTCTCATCCAACAGCCCCTACTTCCAGA
ACAAGTACCTCCCTCATGA

Open reading frame-protein sequence

E X V N X P K Q W Y Q R R V A I **M** A G N T D G T F I I N A H D G N L T **M** T K S
I P S P **M** K F T L L I R A D Q E D **M** A Q Y S V T Q A I V E A R S V T G N P L Q F
S Q S L Y Y G T V V L G S E A G T A V K D K T F P S E I L R I Q A Q Y P G F P D
L N S A V T Y R V T N S S E F **M** **M** N K D I **M** L T A V P **M** E E A R T I R V E V E
A S N T V T K D T A T A V V E I Q V S E R E L P S T E A G G T T G P S S N T T **M**
E A P L T S G T S Q R P A T T S S G G S V G P F P P G G T T L R P P T P A S S I P
G G S P T L G T S T S P Q T T T P G G D S A Q T P K P G T S H P T A P T S R T S T
S L **M**

Clone 11 Casein Kinase II, alpha subunit 2, polypeptide (Mus musculus)

Accession no NM_009974, This clone was fished once.

This has sequence similarity from 411 bp to 1395 bp

ACGCCGAGGTGAACAGCCTGAGGAGCCGCGAGTACTGGGACTACGAAGCCC
ACGTCCCGAGCTGGGGTAATCAAGATGATTACCAACTGGTTCGAAAACTTGG
TCGGGGCAAGTATAGTGAAGTATTTGAGGCCATTAACATCACCAACAATGAG
AGGGTGGTTGTAAAAATTCTCAAGCCAGTGAAGAAAAAGAAGATAAAACGA
GAGGTTAAGATTCTGGAGAACCTTCGTGGTGGAACAAATATCATTAAGCTGA
TTGACACTGTAAAGGACCCTGTGTCAAAGACACCAGCTTTGGTATTTGAATAT
ATCAATAATACAGATTTTAAGCAACTCTACCAGATCCTGACTGACTTTGATAT
CCGGTTTTATATGTATGAACTACTTAAAGCTCTGGATTACTGCCACAGCAAGG
GAATCATGCACAGGGATGTGAAACCTCACAATGTCATGATAGATCACCAACA
AAAAAAGCTCCGACTGATTGATTGGGGTCTGGCAGAGTTCTATCATCCTGCTC
AGGAGTACAATGTTCGAGTGGCCTCGAGGTACTTCAAGGGACCAGAGCTCCT
TGTGGACTATCAGATGTATGATTATAGCTTGGACATGTGGAGCTTGGGCTGCA
TGTTAGCGAGCATGATATTCCGAAAGGAGCCATTCTTCCACGGGCAGGACAA
CTATGACCAGCTTGTTCGAATTGCCAAGGTTCTGGGGACAGATGAACTATAT
GGTTATCTGAAGAAGTACCACATAGACCTAGATCCACACTTCAATGATATCCT
GGGACAACATTCACGGAAGCGCTGGGAAAACTTTATCCATAGTGAGAACAGG
CACCTTGTCAGCCCGGAGGCCCTAGATCTTCTTGACAAGCTCCTGCGGTACGA
CCATCAACAGAGATTGACCGCCAAAGAGGCCATGGAGCACCCATATTTCTAC
CCGGTGGTGAAGGAGCAGTCCCAGCCTTGTGCTGAGAACACCGT

Open reading frame-protein sequence

A E V N S L R S R E Y W D Y E A H V P S W G N Q D D Y Q L V R K L G R G K Y
S E V F E A I N I T N N E R V V V K I L K P V K K K K I K R E V K I L E N L R G G
T N I I K L I D T V K D P V S K T P A L V F E Y I N N T D F K Q L Y Q I L T D F D
I R F Y **M** Y E L L K A L D Y C H S K G I **M** H R D V K P H N V **M** I D H Q Q K K L
R L I D W G L A E F Y H P A Q E Y N V R V A S R Y F K G P E L L V D Y Q **M** Y D
Y S L D **M** W S L G C **M** L A S **M** I F R K E P F F H G Q D N Y D Q L V R I A K V L
G T D E L Y G Y L K K Y H I D L D P H F N D I L G Q H S R K R W E N F I H S E N

R H L V S P E A L D L L D K L L R Y D H Q Q R L T A K E A **M** E H P Y F Y P V V K E Q S Q P C A E N T

Clone 12 Bone morphogenetic protein 1 (BMP1), mRNA

Accession no NM_009755.2, This clone was fished once.

This has sequence similarity from 308 bp to 1196 bp

ACTTGGCCGACTACACCTACGACCTGGGCGAGGAGGACGCCCCGGAGCTCCT
CAACTACAAAGACCCTTGCAAGGCGGCTGCCTTCCTTGGGGACATTGCCCTG
GATGAGGAGGACTTGAGGGCCTTCCAGGTGCAGCAGGCTGCAGTTCTCAGAC
AGCAAACAGCCCGAAGGCCATCCATCAAAGCTGCAGGAAACTCTTCTGCCCT
GGGTGGTCAGGGCACTAGTGGACAGCCGCAGAGGGAAAGCAGGGGCAGATG
GAGAGGCAGGCCTCGGAGCAGGCGGGCAGCGACGTCCAGACCGGAGCGGGT
GTGGCCCGATGGGGTCATCCCGTTTGTGATTGGAGGGAATTTCACCGGCAGC
CAGAGGGCAGTCTTCCGGCAGGCCATGAGACACTGGGAGAAGCATACCTGTG
TCACCTTCTTGGAGCGCACAGATGAGGACAGCTATATTGTATTCACCTACCGA
CCCTGCGGGTGCTGCTCCTACGTGGGTCGCCGAGGTGGGGGCCCCCAGGCCA
TCTCCATCGGCAAGAACTGTGACAAGTTTGGCATCGTGGTCCATGAGCTGGG
CCATGTCATTGGCTTCTGGCACGAGCACACGCGGCCCGACCGGGACCGCCAT
GTCTCTATTGTACGCGAGAACATACAGCCAGGGCAGGAGTATAACTTCTTGA
AGATGGAGGTTCAAGAAGTGGAGTCCTTGGGAGAGACCTATGACTTTGACAG
TATCATGCACTATGCCCGGAACACATTCTCCAGGGGCATCTTCTTGGACACCA
TTGTTCCCAAGTATGAGGTGAATGGGGTGAAGCCTTCCATTGGCCAAAGGAC
CCGACTCAGCAAGGGGGACATCGCTCAGGCCCGGAAGCTCTACAAATGCCCA
GCCTG

Open reading frame-protein sequence

L A D Y T Y D L G E E D A P E L L N Y K D P C K A A A F L G D I A L D E E D L
R A F Q V Q Q A A V L R Q Q T A R R P S I K A A G N S S A L G G Q G T S G Q P
Q R E S R G R W R G R P R S R R A A T S R P E R V W P D G V I P F V I G G N F T
G S Q R A V F R Q A **M** R H W E K H T C V T F L E R T D E D S Y I V F T Y R P C
G C C S Y V G R R G G G P Q A I S I G K N C D K F G I V V H E L G H V I G F W H
E H T R P D R D R H V S I V R E N I Q P G Q E Y N F L K **M** E V Q E V E S L G E T

Y D F D S I **M** H Y A R N T F S R G I F L D T I V P K Y E V N G V K P S I G Q R T R L S K G D I A Q A R K L Y K C P A

Clone 13 Hypothetical protein, Mus musculus coiled-coil domain containing 56 (Ccdc56), mRNA

Accession no AK019025, This clone was fished once.

This has sequence similarity from 1bp to 707bp

AGTGGCCATTATGGCCGGGGGACGGCTTAGGGCGGAGCAGCAACATGGCGG
CCCCTGGAGCTGGTGACCCTCTGAATGCTAAGAATGGAAATGCTCCATTCGCT
CAGCGCATCGACCCGTCGCGGGAGAAATTGACCCCAGCGCAGTTGCAGTTTA
TGCGGCAGGTGCAACTTGCCCAGTGGCAGAAAACACTGCCACAGCGGCGGA
CCCGGAACATCATGACCGGCCTGGGCATCGGGGCCCTGGTGTTAGCTATTTAT
GGTTACACCTTCTACTCGGTGGCCCAAGAGCGTTTCCTTGATGAGCTGGAAGA
TGAAGCCAAAGCTGCCCGAGCCCGTGCTCTTGAGAGAGAGCGAGCTTCGGGA
CCCTAACTGTATGGACCATCTTCAAACTGCTGGATCCCCTGTATATGTTGAAT
GATGCCTGGTGGTCTTACAGCATCACTGGCACACTAACGTCGGACTGTGTGAT
TGAGCCCAAGAAGCCAGAGACTATGCATTTGGCCGCTGCCAAGGGGGGGATT
GCTCACTTCACGTGCAGTTTGTCCAGTTACTCAGCCCTTTTTATTTCCTTGTTT
GAGAGGTATGCAACCTTGCCTTTACCCAAACTTTGTGGTTTACCCCTTCACCT
CCCAGGGGCCCCGACTCTTNAAGGGGANGGGTGTGGCTTNACTGCAGCGTTG
GAACCTTAAAGTGGGAAAGATAGGGTCAAGTTAAAGTAATAAATGAGT

Open reading frame-protein sequence

W P L W P G D G L G R S S N **M** A A P G A G D P L N A K N G N A P F A Q R I D P S R E K L T P A Q L Q F **M** R Q V Q L A Q W Q K T L P Q R R T R N I **M** T G L G I G A L V L A I Y G Y T F Y S V A Q E R F L D E L E D E A K A A R A R A L E R E R A S G P **Stop** L Y G P S S N C W I P C I C **Stop M M** P G G L T A S L A H **Stop** R R T V **Stop** L S P R S Q R L C I W P L P R G G L L T S R A V C P V T Q P F L F P C L R G **M** Q P C L Y P N F V V Y P F T S Q G P R L X K G X G V A X L Q R W N L K V G K I G S S **Stop** S N K **Stop**

Clone 14 Mus musculus integral membrane protein 2A, mRNA

Accession no NM_008409, This clone was fished once.
This has sequence similarity from 127 bp to 891 bp.

TTCAACACCCCTACGGCGGTGCAAAAGGAGGAGGCGCGGCAAGATGTAGAG
GCGCTCGTCAGTCGCACTGTCCGAGCTCAAATCCTGACTGGCAAGGAGCTCA
GAGTTGTCCCGCAGGAGAAAGATGGCTCATCTGGGAGATGCATGCTTACTCT
CCTAGGCCTCTCATTCATCTTGGCAGGACTGATTGTTGGTGGAGCCTGCATTT
ACAAGTACTTCATGCCCAAGAGCACCATTTACCATGGTGAGATGTGCTTCTTT
GATTCTGAGGATCCTGTCAATTCCATTCCTGGAGGAGAGCCATACTTTCTGCC
TGTGACTGAGGAGGCTGATATCCGTGAGGATGACAACATTGCCATCATTGAT
GTGCCTGTGCCCAGTTTCTCTGATAGCGATCCGGCGGCAATTATTCACGACTT
TGAGAAGGGAATGACTGCTTACCTGGACTTGCTTTTGGGAAACTGTTATCTGA
TGCCCCTCAATACTTCCATTGTTATGACTCCAAAGAATCTGGTGGAACTTTTT
GGAAAACTGGCAAGTGGCAAGTATTTGCCTCATACTTATGTGGTTCGTGAAG
ACCTGGTTGCTGTGGAAGAAATTCGTGATGTTAGTAACCTTGGTATTTTTATT
TACCAACTTTGCAACAACCGAAAATCCTTCCGCCTTAGACGCAGAGACCTTCT
GCTGGGTTTCAACAAGCGTGCCATTGACAAATGCTGGAAGATTAGACACTTC
CCCAATGAATTTATCGTTGAAACCAAGATC

Open reading frame-protein sequence

F N T P T A V Q K E E A R Q D V E A L V S R T V R A Q I L T G K E L R V V P Q
E K D G S S G R C **Met** L T L L G L S F I L A G L I V G G A C I Y K Y F **Met** P K S
T I Y H G E **Met** C F F D S E D P V N S I P G G E P Y F L P V T E E A D I R E D D N
I A I I D V P V P S F S D S D P A A I I H D F E K G **Met** T A Y L D L L L G N C Y L
Met P L N T S I V **Met** T P K N L V E L F G K L A S G K Y L P H T Y V V R E D L
V A V E E I R D V S N L G I F I Y Q L C N N R K S F R L R R R D L L L G F N K R
A I D K C W K I R H F P N E F I V E T K I

Clone 15 Mus musculus Putative tumor differentially expressed 2 or serine incorporator 1

Accession no AK141574, This clone was fished once.
This has sequence similarity from 1270bp to 2042 bp

GGAGGCNGTGAATTCMCCCAAGCAGTGGTATCAACGCAGAGTGGCCATTATG
GCCGGGGGATGGAGTCACGTACAGTTACTCCTTCTTTCACTTCATGCTCTTCT
TGGCTTCACTTTACATCATGATGACCCTCACCAATTGGTACAGGTATGAGCCT
TCTCGTGAGATGAAAAGTCAGTGGACAGCAGTCTGGGTGAAAATCTCTTCCA
GTTGGATTGGCCTTGTGCTGTACGTGTGGACACTAGTGGCACCACTTGTTCTT
ACAAATCGGGATTTTGACTGAATGGGATGTCTGGCATGAGTCCCACTTTGGCC
ATTGTCTATTTGACAGTAACAGTATTCCCAATTTTTGTAAAGTTGTATGTGCT
GTTGTGTAAGTGTAACACTGTTTTTCTCTTGCATGAAATAGATTTACTCTGCC
AATTTTATCCATGTTCTTGCTAAGTCTATTGATGATATGAATTGGAAAGGGAG
AATTTTGAATATGGTGAGAAATTGGGAAAAATGGACATTGATAGGCATATCA
TCTGCTTTGTGTACATATGGTATTAAAACTGAAAACAAATATTCTTGACATTT
GGGTTTCAAAATTGTGTTAGCACTTATTTTAGAAACATTAATTAAGTGTATGG
CTGCCTTTGAAATACTTGATGTGTTGTTGTTGCCTTACAGAGGACTGCAAGTA
TAAAGCAAATAGCTGTTTTAAAATTATGTAAATAAGTCGCAGGCTAGTTATTT
GAAAACTCTAAGTCACTTTAATTTTGTCAGAATCTTCTCTTACTAGGTAGGCC
CAGGATGTTCCATAGTTAGTGGAAAACAGTACAGGAGG

Open reading frame-protein sequence

R X **Stop** I X P S S G I N A E W P L W P G D G V T Y S Y S F F H F **M** L F L A S L
Y I **M** **M** T L T N W Y R Y E P S R E **M** K S Q W T A V W V K I S S S W I G L V L
Y V W T L V A P L V L T N R D F D **Stop** **M** G C L A **Stop** V P L W P L S I **Stop** Q
Stop Q Y S Q F L **Stop** S C **M** C C C V S V T L F F S C **M** K **Stop** I Y S A N F I H V L
A K S I D D **Met** N W K G R I L N **M** V R N W E K W T L I G I S S A L C T Y G I K
T E N K Y S **Stop** H L G F K I V L A L I L E T L I K C **M** A A F E I L D V L L L P Y
R G L Q V **Stop** S K **Stop** L F **Stop** N Y V N K S Q A S Y L K T L S H F N F V R I F S
Y **Stop** V G P G C S I V S G K Q Y R R

Clone 16 SMAD specific E3 ubiquitin protein ligase 2, transcript variant 2 (smurf2), mRNA

Accession no XM_001001809, This clone was fished once.

This has sequence similarity from 4233bp to 4984bp.

AGGCNGTGAATTCNCCCAAGCAGTGGTATCAACGCAGAGTGGCCATTATGGC
CGGGGGGAGGCAGAGGCAGAAGCGGCAGCATGCCAAGGCCAGTCTCACCTA
CACAGTGGATTGAGGCTTCCTTGAGACCCTGTCTCAGAGGACAGGGATGCAG
TGCTAAGGAGACGCTGCTTTCAGTGGTTTTCCATGCAGTGTAACACAGGCAA
ATCCCACTGCTTTCTGAGTGCCTTTTAACTTTATTACAGTGACTTGACAGAGC
TTTAGAGGAAACTGCCCTATGTAACGGTCTTCAATAGCACAGACATCAAAAA
CATGGTTTAGATTTCGATTTCAAAATTACATTACCTGATTACTTCCAAATAGG
TGCTAAGCAAACAAAAGTCCCCAGTGTCACAGAACCTGGACTTGCCTTGTGT
TTAATGTGAATATTTATCTGTCCCCACGTCGCCTGCCTCCAGGCGCTCTGTTCC
ATCAGGAAAGCTTCATTCTGACCAATGGTTCCCTGTGCTCACATACAGTAAGA
GGCCACTATTTTCTGGCACAAAGTTTTAAAGCAGTGCTGTGTATCCTGTACAT
TTGTTTTGCAGTTTGTTGCTCTGTATTTTACAGTGCTCTCCCCACCCCACTCAC
ATTTACAGGCCAGAGCACAAATTTCTCTTCTCCTAAATCCACTATAATTTGCT
TTCCTAGACAACACTAAATCTGCCCTGAGCTTGCAGTGCATCGGACAGATGG
CACTGGTGGGTGTTAGCCGCTTCCAGTGCCTGCAGGGTCAGCAGCACAGCCT
CGCCCTTCAGCTGCAGCTT

Open reading frame-protein sequence

R X **Stop** I X P S S G I N A E W P L W P G G G R G R S G S **M** P R P V S P T Q W I E A S L R P C L R G Q G C S A K E T L L S V V F H A V **Stop** H R Q I P L L S E C L L T L L Q **Stop** L D R A L E E T A L C N G L Q **Stop** H R H Q K H G L D F D F K I T L P D Y F Q I G A K Q T K V P S V T E P G L A L C L **M Stop** I F I C P H V A C L Q A L C S I R K A S F **Stop** P **M** V P C A H I Q **Stop** E A T I F W H K V L K Q C C V S C T F V L Q F V A L Y F T V L S P P H S H L Q A R A Q I S L L L N P L **Stop** F A F L D N T K S A L S L Q C I G Q **M** A L V G V S R F Q C L Q G Q Q H S L A L Q L Q L

Clone 17 Mus musculus Tp53rk binding protein (TprKb), mRNA

Accession no NM_176842, This clone was fished once.

This has sequence similarity from 281 bp to 797 bp

ATCACAGCAGCTGGACTTGTTTCCTGAATGCAGGGTGACCCTTCTGTTATTTA
AAGATGTAAAAAATGCAGGAGACTTGAGAAAAAAGGCCATGGAAGGATCTA

TTGATGGTTCATTGATAAACCCCAATGTGATTGTCGATCCATTTCAGATACTT
GTGGCAGCAAACAAAGCCGTTCATCTCCACAGACTGGGAAAAATGAAGACA
AGAACTCTGTCTACTGAAATTATCTTCAACCTCTCCCCAAATAATAATATTTC
AGAGGCCTTGAAGAAATTTGGCATTTCAGAAACTAACACTTCAGTTCTGATTG
TTTACATTGAAGATGGAAGCAAACAAGTACCACAGGAACACCTAGTATCTCA
AGTGGAAGGCCAGCAGGTGCCTTTGGAAAGTCTTCCAGAAATAACAAGGCTC
TCGGAAGTCAAGAAGATATATAAACTGTCATCACAAGAAGAGAGGATTGGG
ACGTTATTGGATGCTATCATTTGTAGAATGTCAACGAAGGATGTTTTA

Open reading frame-protein sequence

S Q Q L D L F P E C R V T L L L F K D V K N A G D L R K K A **M** E G S I D G S L I
N P N V I V D P F Q I L V A A N K A V H L H R L G K **M** K T R T L S T E I I F N L
S P N N N I S E A L K K F G I S E T N T S V L I V Y I E D G S K Q V P Q E H L V S
Q V E G Q Q V P L E S L P E I T R L S E V K K I Y K L S S Q E E R I G T L L D A I I
C R **M** S T K D V L

Clone 18 SMC 4 structural maintenance of chromosomes 4 - like 1

Accession no NM_133786, This clone was fished once.

This has sequence similarity from 2907bp to 3649bp

GTGGTATCAACGCANAGTGGATTATGGCCGGGGGATTCTGTTTGCCGCACAG
AGAAAGAAATAAAAGACACTGAAAAAGAAATAAATGACTTAAAGACAGAGC
TAAAAAATATTGAAGACAAGGCAGAAGAGGTCATAAACAATACAAAAACTG
CAGAGACATCCTTACCAGAAATCCAGAAAGAACATCGTAATCTACTTCAGGA
ACTAAAAGTTATTCAAGAAAATGAACATGCTCTTCAGAAAGATGCACTTAGT
ATTAAGTTGAAACTTGAACAGATAGATGGCCACATTTCTGGACATAACTCAA
AAATAAAATACTGGCAAAAAGAGATTTCAAAAATAAAATTGCATCCTGTTGA
AGATAATCCTGTTGAGACAGTAGCAGTTCTAAGCCAGGGGGAGCTTGAGGCA
ATCAAGAATCCAGAGTCTATAACAAATGAAATTGCACTTTTGGAAGCTCAGT
GTCGTGAAATGAAACCAAACCTTGGAGCCATCGCTGAGTATAAAAAAAAGG
AAGATTTATATTTGCAAAGAGTAGCCGAACTGGACAAAATTACTTCTGAAAG
AGATAATTTTAGACAAGCATATGAAGATCTTCGAAAACAAAGGCTGAATGAA

TTTATGGCTGGTTTTTACGTAATAACAAATAAACTAAAAGAAAACTACCAGA
TGCTCACATTGGGAGGAGATGCTGAACTGGAGCTTGTGGACAGTTTAGATCC
TTTTTCTGAAGGAATCATGTTCAGTGTTCGGCCACCTAAGAAAAGTTGGAGA

Open reading frame-protein sequence

V V S T X S G L W P G D S V C R T E K E I K D T E K E I N D L K T E L K N I E D
K A E E V I N N T K T A E T S L P E I Q K E H R N L L Q E L K V I Q E N E H A L
Q K D A L S I K L K L E Q I D G H I S G H N S K I K Y W Q K E I S K I K L H P V E
D N P V E T V A V L S Q G E L E A I K N P E S I T N E I A L L E A Q C R E **M** K P
N L G A I A E Y K K K E D L Y L Q R V A E L D K I T S E R D N F R Q A Y E D L
R K Q R L N E F **M** A G F Y V I T N K L K E N Y Q **M** L T L G G D A E L E L V D S
L D P F S E G I **M** F S V R P P K K S W R

Clone 19 Mus musculus transmembrane protein 45b (Tmem45b), mRNA

Accession no NM_144936, This clone was fished once.

This has sequence similarity from 57 bp to 872 bp

CAAGGGCCATGCTCTCCCAGGGAGCTTCTTTCTAATAGTGGGGCTGTGGTGGT
CAGTGAAGTACCCACTGAAGTACTTTCACCACAAGGGCTTGAAGAATAACAG
ACTGAGCCGTCAGCAGGAGCGCATTGAGATCATTGAAGGTGCAGTGAAGACT
CTGTTTGCAATCATTGGGATCCTGGCAGAGCAGTTTGTTCCAGATGGACCTCA
CCTGCACCTCTACCACGAGAACCAATGGGTCAAGCTGATGAATTGGCAGCAC
AGCACCATGTACCTATTCTTCGGTGTCTCGGGACTCATGGACATGATCACCTA
CCTTTACTTCCACATCGTGCCCCTGGGGTTAGACAGAGTGGTTTTGGCTATGG
CCGTATTCATTGAAGGTTTTCTCTTCTACTTCCACGTTCACAACCGCCCTCCCC
TGGACCAGCACATCCACTCCCTGCTGCTGTTCGGTCTTTTCGGAGCAGCTGTC
AGCATCTCTCTGGAGGTTATCCTTCGGGATAACATCGTGCTGGAGCTCTTCCG
AACCAGTCTCCTTATCTTGCAGGGAACCTGGTTCTGGCAGATTGGATTTGTCC
TGTTCCCGCCATTTGGAAGGCCAGAATGGGACCAGAAGGACATGGACAACAT
CATGTTCATCACCATGTGCTTCTGCTGGCACTACTTGGTTGCTCTCTGCATCGT
AGCCATCAACTACTCCCTTGTTTACTGCTTTCTGACTCGGGTGAAGAGACGTG
CAGAAGGAGAAATCATTGGGATTCAGAAGCTGAAGTCTGATCACACTTACCA
GTCAGCCCTTTTGAGTGGCTCGGA

Open reading frame-protein sequence

K G H A L P G S F F L I V G L W W S V K Y P L K Y F H H K G L K N N R L S R Q
Q E R I E I I E G A V K T L F A I I G I L A E Q F V P D G P H L H L Y H E N Q W V
K L **Met** N W Q H S T **Met** Y L F F G V S G L **Met** D **Met** I T Y L Y F H I V P L G L
D R V V L A **Met** A V F I E G F L F Y F H V H N R P P L D Q H I H S L L L F G L F
G A A V S I S L E V I L R D N I V L E L F R T S L L I L Q G T W F W Q I G F V L F
P P F G R P E W D Q K D **Met** D N I **Met** F I T **Met** C F C W H Y L V A L C I V A I N
Y S L V Y C F L T R V K R R A E G E I I G I Q K L K S D H T Y Q S A L L S G S

Clone 20 Mus musculus similar to prochymosin, transcript variant 1 (LOC634449), mRNA

Accession no XM_987135, This clone was fished once.

This has sequence similarity from 6 bp to 821 bp

GTGGCCATTTCCTGTGATCCAGAATGAGGCGCTTTGTGCTGCTCCTCGCTGCT
CTCGCCATCTCCCAGAGCCATGTGGTCACCAGGATCCCCCTGCACAAAGGGA
AGTCTCTGAGGAACACCCTGAAGGAGCAAGGACTGCTGGAGGACTTTCTGAG
CAGACAGCAGTACGAGTTCAGTGAGAAGAACTCCCGCATCGGGGTGGTGGCC
AGTGAGCCTCTGATCAACTATCTAGATAGTGAGTACTTTGGAACGATCTACAT
TGGGACACCACCGCAGGAGTTCACCGTGGTGTTTGACACAGGCTCCTCAGAA
CTCTGGGTACCCTCTGTCTACTGCAACAGCAAAGTGTGCCGAAACCACCACC
GTTTTGACCCATCCAAGTCCATCACCTTCCAGAACCTGAGCAAGCCCCTGTTT
GTCCAGTATGGTACTGGTAGAATGGAGGGCTTCCTGGCCTACGACACTGTCA
CAGTCTCTGATATTGTAGTGTCCCATCAGACTGTGGGCCTGAGTACCCAGGAA
CCGGGCGACATCTTCACCTACTCTCCATTTGATGGCATCCTGGGCCTGGCCTA
TCCTACTTTTGCCTCCAAATACTCAGTACCCATATTTGACAACATGATGAACA
GGCACCCGGTGGCCCAAGACCTGTTCTCCGTTTACATGAGCAGGAATGAGCA
GGGGAGCATGCTCACACTGGGGGCCATCGATCAGTCCTACTTCATAGGCTCA
CTGCACTGGGTGCCTGTGACAGTACAGGGATATTGGCAGTTCACAGTGGACA
GGATCACAATCAATGGTGAAGTGGTGGCTTGT

Open reading frame-protein sequence

G H F L **Stop** S R **M** R R F V L L L A A L A I S Q S H V V T R I P L H K G K S L R
N T L K E Q G L L E D F L S R Q Q Y E F S E K N S R I G V V A S E P L I N Y L D
S E Y F G T I Y I G T P P Q E F T V V F D T G S S E L W V P S V Y C N S K V C R
N H H R F D P S K S I T F Q N L S K P L F V Q Y G T G R **M** E G F L A Y D T V T V
S D I V V S H Q T V G L S T Q E P G D I F T Y S P F D G I L G L A Y P T F A S K Y
S V P I F D N **M** **M** N R H P V A Q D L F S V Y **M** S R N E Q G S **M** L T L G A I D Q
S Y F I G S L H W V P V T V Q G Y W Q F T V D R I T I N G E V V A C

Clone 21 ATPase, H+ transporting, lysosomal V1 subunit G1 [Mus musculus]

Accession no AK088712, This clone was fished once.

This has sequence similarity from 14 bp to 826 bp

GTGGCCATTATGGGGGGGATCCGAGGGGCTTCGGAGGGTGTTTGAGCAGTTG
ACTTAGGCCGTTCTCTCGGCCCAAAGTCCCCTCAGCAATGGCGAGTCAGTCG
CAGGGCATCCAGCAGCTACTGCAGGCCGAGAAGCGGGCCGCCGAGAAGGTG
TCCGAGGCCCGCAAGCGAAAGAACCGGAGGCTGAAGCAGGCCAAAGAAGAA
GCCCAGGCTGAAATTGAACAGTACCGCCTGCAGAGGGAGAAGGAGTTCAAG
GCCAAGGAAGCTGCGGCATTGGGCTCCCATGGCAGCTGCAGCAGTGAGGTGG
AAAAGGAGACCCGGGAGAAGATGACCGTCCTCCAGAACTACTTCGAGCAGA
ACAGGGATGAAGTCCTGGATAACCTCTTGGCCTTTGTGTGTGACATCCGGCCG
GAAATCCATGAAAACTACCGCATAAACGGATAGGAGGTGGAAGCGCCTGCTC
TGTGGGTTGGCATGAGATCTGCCCCCATAGAATAGGAAAACCTCCACAGAGC
TGGAGTTCTGTTCTGGAAGGCATTAAATTATTTCTCTATGCTGTTGTAGGACC
CTTCACTTTTTGAGAGTATCAAATCTAGCTTTTTGTACAGACTTCTTATGTACC
TGAAGTTTCATCTTCTTATCTTGTGTGTCTTTAAGGAGTGTATGTCGCCTGTTT
TCCTGTGTCTTCATGCAAGTAATCGATATCAATGTTGACTTTTTTTTTTTAATT
AGTCTAGTAGGAAAAATTCTTTGAAGGAAGAAGGGGAGTAAACTGTTCCCTT
CTCCTTTCTTACAGGTCAGGGGCGTTATCTATGAAAAAGTAGAAGT

Open reading frame-protein sequence

G H Y G G D P R G F G G C L S S **Stop** L R P F S R P K V P S A **Met** A S Q S Q G I Q Q L L Q A E K R A A E K V S E A R K R K N R R L K Q A K E E A Q A E I E Q Y R L Q R E K E F K A K E A A A L G S H G S C S S E V E K E T R E K **M** T V L Q N Y F E Q N R D E V L D N L L A F V C D I R P E I H E N Y R I N G **Stop** E V E A P A L W V G **M** R S A P I E **Stop** E N L H R A G V L F W K A L N Y F S **M** L L **Stop** D P S L F E S I K S S F L Y R L L **M** Y L K F H L L I L C V F K E C **M** S P V F L C L H A S N R Y Q C **Stop** L F F F N **Stop** S S R K N S L K E E G E **Stop** T V P F S F L T G Q G R Y L **Stop** K S R S

Clone22 Eukaryotic translation initiation factor 3, subunit 8 (Eif3s8) Pseudogene similar to stathmin1 (Stmn1)

Accession no AL845317, This clone was fished once.

This has sequence similarity from 20 bp to 467 bp

AGTGTGGCCAGGCGGCTCGGACCGAGCAGGGCTTTCCTTGCCAGTGGATTGT
GTAGAGTATACAGCCAGTCTCTTGTCTTCTGTTCGACATGGCATCTTCTGATA
TTCAGGTGAAAGAGCTGGAGAAGCGCGCTTCAGGCCAGGCTTTTGAGCTGAT
TCTCAGCCCTCGGTCAAAAGAATCTGTCCCCGATTTCCCCCTTTCCCCCCCAA
AGAAGAAGGACCTTTCCCTGGAGGAAATCCAGAAGAAATTAGAAGCTGCAG
AAGAAAGGCGCAAGTCTCATGAGGCGGAAGTCTTGAAGCAGCTCGCGGAGA
AGCGGGAGCATGAGAAGGAGGTGCTCCAGAAAGCCATCGAGGAGAACAACA
ACTTCAGCAAGATGGCGGAGGAGAAGCTGACCCACAAAATGGAGGCTAACA
AAGAGAACCGGGAGGCGCAGATGGCGGCCAAGCTGGAGCGCTTGCGAGAGA
AGGACAAGCACGTGGAAGAGGTGCGGAAGAACAAAGAATCCAAAGACCCCG
CGGATGAGACTGAGGCTGACTAAGTTGTCCCGAGAACTGACTTTCTCCCCGA
CCCCGTCCTAAATATCCAAAGACTGTACTGGCCAGTGTCCTTTACTTTCCCTC
CTGACAGATAGTCTAGAAGCCGATGTAGGACCGTATAGGTAGATCCAGACCG
TGAGATGTTTTAGGGGCTCAAGGGGAGAAACTGAAAGTGTTTTACTCTTTTTT
TAAAGTGTTGGTCTTTCTAATGTAGCTATTTTTCTCGTTGCATCTTTTCCACTC
GGGCACAATCGGTGTGCTGGGTTAATGG

Open reading frame-protein sequence

C G Q A A R T E Q G F P C Q W I V **Stop** S I Q P V S C L L F D **M** A S S D I Q V K E L E K R A S G Q A F E L I L S P R S K E S V P D F P L S P P K K K D L S L E E I Q K K L E A A E E R R K S H E A E V L K Q L A E K R E H E K E V L Q K A I E E N N N F S K **M** A E E K L T H K **M** E A N K E N R E A Q **M** A A K L E R L R E K D K H V E E V R K N K E S K D P A D E T E A D **Stop** V V P R T D F L P D P V L N I Q R L Y W P V S F T F P P D R **Stop** S R S R C R T V **Stop** V D P D R E **M** F **Stop** G L K G R N **Stop** K C F T L F L K C W S F **Stop** C S Y F S R C I F S T R A Q S V C W V N

Clone 23 Similar to SH3-domain GRB2-like B1 (endophilin) [Mus musculus]

Accession no XM_988661, This clone was fished once.

This has sequence similarity from 691 bp to 1368 bp

TTAAATTTCCTCACTCCTTTAAGAAACTTTATAGAAGGGGATTACAAAACAAT
CGCAAAAGAAAGGAAACTATTACAGAATAAGAGACTGGATTTGGATGCTGC
AAAAACAAGACTAAAAAAGGCAAAAGCTGCAGAAACTAAAAGTTCATCTGA
ACAGGAATTGAGAATAACTCAAAGTGAATTTGATCGTCAGGCAGAGATTACC
CGACTCCTGCTTGAGGGAATCAGCAGTACACACGCCCATCATCTCCGCTGTCT
GAATGACTTTGTAGAAGCCCAGATGACTTACTATGCACAGTGTTACCAGTAT
ATGCTAGACCTACAGAAGCAACTGGGAAGTTTTCCATCCAATTATCTTTCTAA
CAACAATCAGACCTCTGGGACACCAGTGCCATATGCTTTGTCAAATGCAATT
GGTCCTTCTGCCCAGGCTTCAACGGGTAGCCTTGTAATCACCTGTCCTTCTAA
CCTCAATGACCTTAAAGAATCCAGCAACAACAGGAAGGCTAGGGTCCTCTAT
GATTATGATGCTGCAAATAGCACTGAACTGTCACTCCTGGCCGATGAGGTAA
TCACTGTGTTCAGTGTCGTTGGAATGGACTCCGACTGGCTAATGGGAGAGAG
AGGAAATCAAAAGGGCAAGGTGCCAATTACCTACTTAGAACTTCTCAATTAA

Open reading frame-protein sequence

L N F L T P L R N F I E G D Y K T I A K E R K L L Q N K R L D L D A A K T R L K K A K A A E T K S S S E Q E L R I T Q S E F D R Q A E I T R L L L E G I S S T H A H H L R C L N D F V E A Q **M** T Y Y A Q C Y Q Y **M** L D L Q K Q L G S F P S N Y

L S N N N Q T S G T P V P Y A L S N A I G P S A Q A S T G S L V I T C P S N L N
D L K E S S N N R K A R V L Y D Y D A A N S T E L S L L A D E V I T V F S V V
G **M** D S D W L **M** G E R G N Q K G K V P I T Y L E L L N **Stop**

Clone 24 Mus musculus amylase 2a2, pancreatic (Amy2a2), mRNA.

Accession no NM_001160152, This clone was fished once.

This has sequence similarity from 172bp to 488bp
TGCTTTCCCTCATTGGGTTCTGCTGGGCTCAATATGACCCACATACTTNAGAT
GGGAGGACTGCTATTGTCCACCTGNTCNAGTGGCGCTGGGTTGATATTGCCA
AGGAATGTGANCGATACTTAGCTCCTAAGGGATTTGGAGNGGTGCNGGTCTC
TCCACCCAATGAAAACGTTGNANTTCATAACCCATCNANANCTTNGTGGGAA
AGATACCAACCAATCANCTATAAAATCTGCACAAGGTCTGGAAATGAAGATG
AATTCANANACATGGNGACAAGGNGCAACAATGTTGGTGTCCGTATTTATGT
GGAT
Open reading frame-protein sequence
L S L I G F C W A Q Y D P H T X D G R T A I V H L X X W R W V D I A K E C X R
Y L A P K G F G X V X V S P P N E N V X X H N P X X X X W E R Y Q P I X Y K I
C T R S G N E D E F X X **M** X T R X N N V G V R I Y V D

3.2 Overexpression and Purification of HAX1

The second goal of this thesis is to purify HAX1 protein to be able to use it for pull down assays, Structural studies and other interaction studies.
Hence the necessity to clone Murine and Human HAX1 cDNA's into protein overexpression vectors was felt to be able to over express and purify the proteins in large quantities.

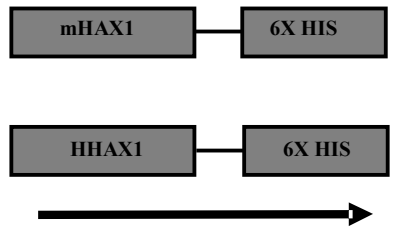
3.2.1 Cloning constructs for expression of HAX1 in E.coli

3.2.1.1 Generation of 6X His fused HAX1 expression constructs.

To generate 3' 6X His tagged mHAX1 protein we have prepared mHAX1-His expression construct by amplifying the cDNA of mHAX1 using primers with restriction sites for

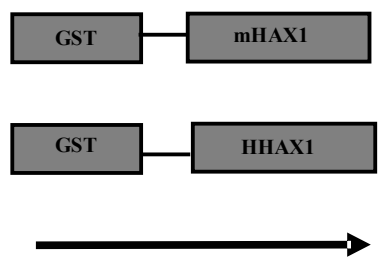
BspH1 and HindIII enzymes. PCR product of mHAX1 was cloned into pGEM-T vector, verified by sequencing and subcloned into the pET24d expression vector using Nco1 and Hind III restriction sites.

Similarly HHAX1 was cloned into Nco1 and EcoR1 sites to generate HHAX1-His expression construct.



3.2.1.2 Generation of GST fused HAX1 expression constructs.

To generate 5' GST tagged mHAX1 and HHAX1 proteins we have prepared GST-mHAX1 and GST-HHAX1 expression constructs by amplifying the respective cDNA's using primers with restriction sites for BamH1 and EcoR1 enzymes. PCR products were cloned into pGEM-T vector, verified by sequencing and subcloned into pGEX4T-1 expression vector using BamH1 and EcoR1 restriction sites.



3.2.1.3 Overexpression of HAX1 fusion constructs in *E.coli*

Murine and Human Hax1 were over expressed in *E.coli* as His-fusion and GST-fusion proteins in different strains of *E.coli* under varying conditions of temperatures.

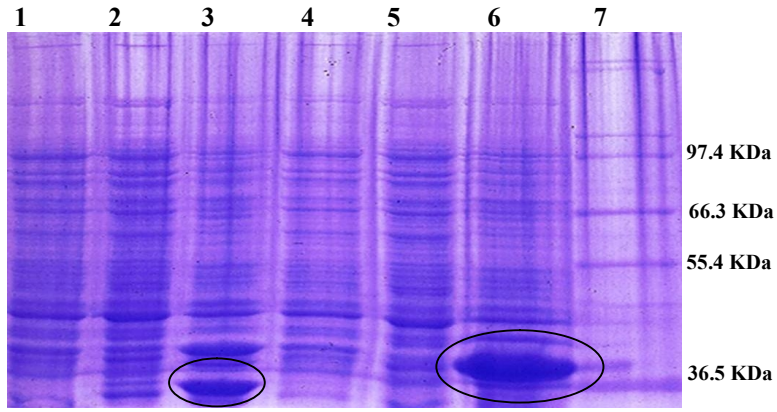


Fig 11: Over expression of mHAX1-His in *E.coli* PLys S. Lane1: 20C Before IPTG Induction, Lane2: soluble fraction after Induction at 20C, Lane 3: Insoluble fraction after Induction at 20C, Lane 4: 37C before Induction, Lane5: soluble fraction after Induction at 37C, Lane 6: Insoluble fraction after Induction at 37C, Lane 7: Protein molecular weight marker. Most of the over expressed protein was observed in the Insoluble fraction.

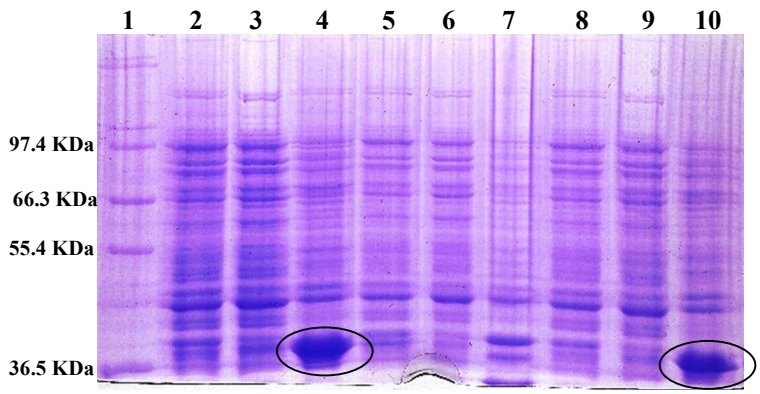


Fig 12: Over Expression of H HAX1-His in *E.coli* P LYS S and DE3 strains. Lane1: Protein molecular weight marker, Lane2: 20C before Induction in DE3, Lane 3: soluble fraction after induction at 20C in DE3, Lane 4: Insoluble fraction after Induction at 20C in DE3, Lane 5: Before induction at 37C in DE3, Lane 6: soluble fraction after induction at 37C in DE3, Lane 7: Insoluble fraction after induction at 37C in DE3, Lane 8: Before induction at 20C in PLYS S, Lane 9: soluble fraction after induction at 20C in PLYS S, Lane 10: Insoluble fraction after Induction at 20C in PLYS S. Most of the protein has been observed in the insoluble fraction.

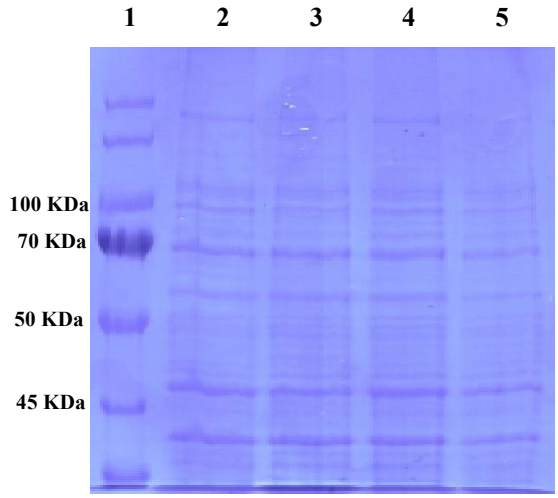


Fig 13: Overexpression of GST-mHAX1 and GST-HHAX1 in *E.coli* BL21 strain. Lane 1: Protein Molecular weight marker, Lane 2: GST-mHAX1 before Induction at 37C, Lane 3: GST-mHAX1 after Induction at 37C, lane 4: GST-HHAX1 before Induction at 37C, Lane 5: GST-HHAX1 after Induction at 37C. There was no observation of any HAX1 expression.

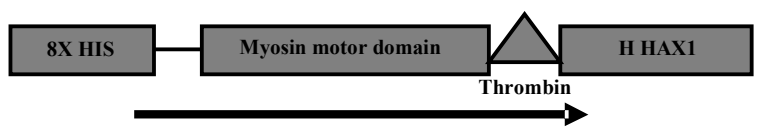
Conclusion: It was observed that both Murine and Human HAX1 proteins go into insoluble, inclusion bodies when over expressed with 6X His tag and there is no observance of expression with GST tag in *E.coli*.

We then came across a cellular slime mold *Dictyostelium discoideum* a eukaryotic model organism for overexpression of recombinant myosin and myosin fused proteins.

3.2.2 Cloning constructs for expression of HAX1 in *Dictyostelium discoideum.*

3.2.2.1 Generation of 8X His tagged Myosin motor domain fused H.HAX1 expression construct.

To generate 5' 8X His-Myosin-thrombin-H.HAX1 protein we have prepared H.HAX1 expression construct by amplifying Human HAX1 cDNA using primers with restriction sites for Sac1 and Not1 enzymes. PCR product was cloned into pGEM-T vector, verified by sequencing and subcloned into pDXA M765 MCS expression vector using Sac1 and Not1 restriction sites.



3.2.2.2 Transformation and screening of ORF$^+$ clones expressing Myosin fused H.HAX1:

H.HAX1-pDXA M765 MCS clone was transformed into *Dictyostelium discoideum* ORF$^+$ cells by electroporation and the cells were plated and cultured in G418 selection medium. The cells were then checked for H.HAX1 expression by coomassie blue stain and western blot. Expected size of Myosin fused H.HAX1 is approximately 125KDa.Western blot was done using HAX1 antibody at a dilution of 1:1000 in 5% milk PBST overnight.

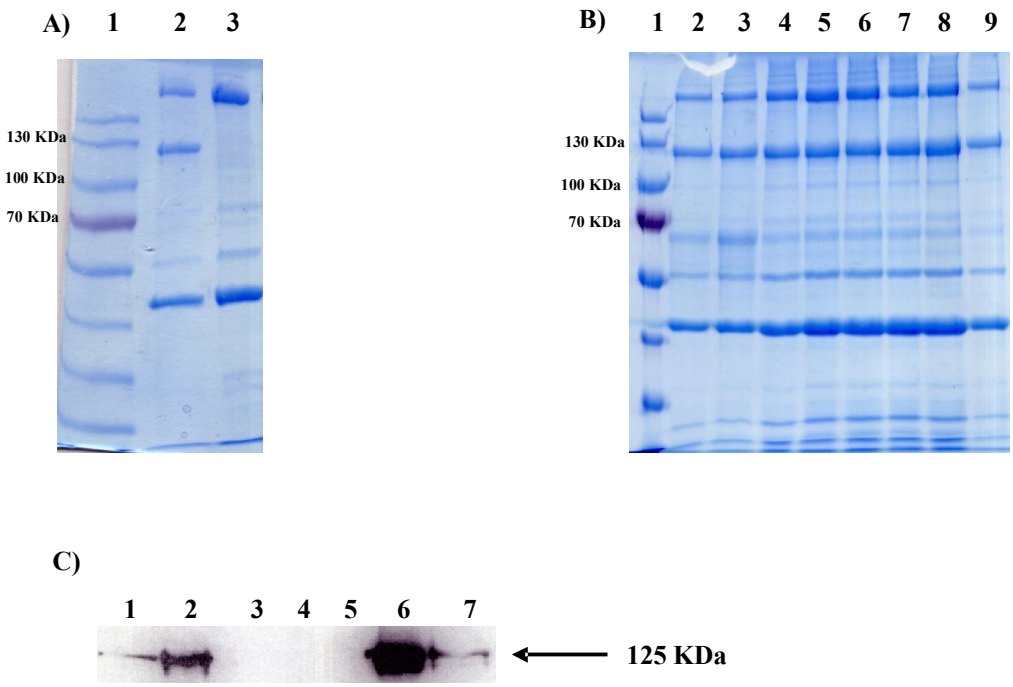


Fig 14: Screening of Transformants.

A) Coomassie blue staining of 10% SDS page gel. Lane 1: Prestained ladder, Lane2: Clone A1 Lysate, Lane3: Lysate from control ***Dictyostelium discoideum*** ORF+ cells.

B) Coomassie blue staining of 10% SDS page gel. Lane 1: Prestained ladder, Lane2: Clone A1 Lysate, Lane3: Clone A2 Lysate, Lane4: Clone A3 lysate, Lane 5: Clone A4 lysate, Lane 6: Clone A5, Lane 7: Clone A6, Lane 8 Clone B1, Lane 9: Clone B2. All the tested clones were observed to express Myosin fused H.HAX1 at the correct size (125KDa).

C) Western blot analysis of clones A1 and A2 using HAX1 antibody (1:1000) Lane 1: Soluble fraction of A1, Lane2: Insoluble fraction of A1, Lane 3: Soluble fraction of ORF$^+$ cells, Lane 5: Insoluble fraction of ORF$^+$ cells, Lane 6: Soluble fraction of A2, Lane 7: Insoluble fraction of A2. Clone A2 was used for all further experiments. Endogenous Myosin and Actin bands were also observed.

3.2.2.3 Purification of Myosin fused H.HAX1

Histidine Tagged Myosin Fused HAX1 protein was purified using AKTA PURIFIER system. Here the cell lysates were loaded on to a Ni-NTA matrix for Affinity purification of HAX1 It was observed that the affinity of the protein to the column is not very high as shown in the figure (15) and so the sample was loaded twice more on the same column to increase the binding efficiency. The sample was analyzed on SDS gel to check for purity after elution with 400 mM Imidazole.

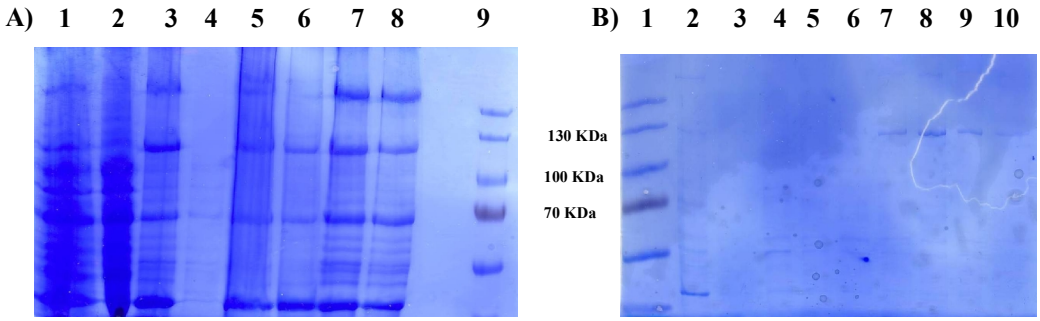


Fig 15: Purification of His-Myosin-H.HAX1. A) Lane1: Total cell lysate, Lane2: supernatant after cell lysis, Lane 3: Pellet after cell lysis, Lane 4; Supernatant before ATP extraction, Lane 5: Pellet before ATP extraction, Lane 6: Pellet after ATP extraction, Lane 7: Supernatant after ATP extraction, Lane 8: Flow through after loading on Ni-NTA column, Lane 9: protein molecular weight marker. **B)** Lane 1: protein molecular weight marker, Lane2: Low salt wash flow through, Lane 3: High salt wash flow through, Lane 4: Low salt buffer with 50 mM Imidazole wash flow through, Lane 5: Fraction A4, Lane 6: Fraction A7, Lane 7: Fraction A14, Lane 8: Fraction B2, Lane 9: Fraction B10, Lane 10: Fraction C1.

The samples from Fractions A10 to C1 were pooled and dialyzed in gel filtration buffer (50mM Tris pH 7.5, 200mM NaCl, 1mM DTT) overnight to remove Imidazole and other salts. The protein was then concentrated and loaded onto a pre equilibrated gel filtration Sephadex-200 column for size exclusion chromatography. The hax1 protein was found to be degraded after gel filtration as shown in the figure (16).

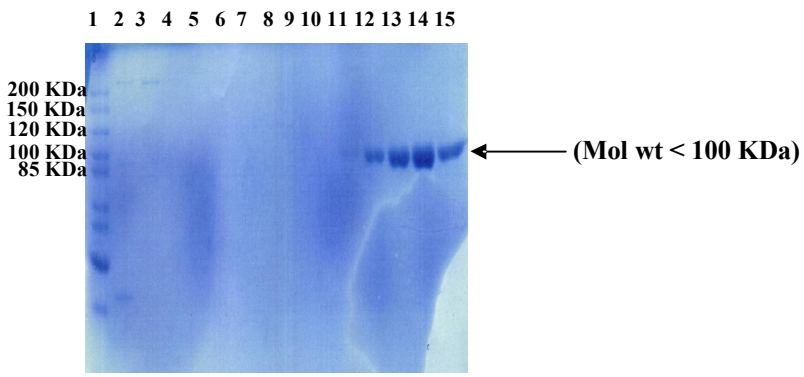


Fig 16: Purification of His-Myosin-h.HAX1 by Gel filtration. Lane 1-10: samples of gel filtration fractions A3 to A13, Lane 11-15: Purified His-Myosin-mHAX1. HAX1 was found to be degraded as can be seen by the shift in the molecular weight.

3.3 Characterization of HAX1 interacting proteins

The third goal of this thesis is to confirm the interactions of proteins fished by the Yeast Two hybrid Screen Using Immunoprecipitation and Colocalization Techniques.

Two proteins a) Similar to SH3 domain GRB2- like protein B1 (Endophilin B1), Accession no XM_988661 and b) Mus musculus serine (or cysteine) peptidase inhibitor, clade B, member 1a, mrna, Accession no BC104333 were selected for further studies because a) Loss of Endophilin b1 was reported to be involved in the suppression of BAX and BAK conformational changes and mitochondrial apoptosis(Yoshinori Takahashi 2005) and it interacts with Beclin-1 through UVRAG and regulated autophagy and tumorigenesis(Takahashi, Coppola et al. 2007), While b) Serpin b1a or Monocyte/ Neutrophil Elastase inhibitor (MNEI) a 42-kD Serpin molecule, is found in the cells (neutrophils, monocytes, and macrophages) that accumulate at inflammatory sites and inhibits Neutrophil elastase, cathepsin G and Proteinase 3 (Remold-O'Donnell, Nixon et al. 1989), (Remold-O'Donnell, Chin et al. 1992).

3.3.1 Cloning of Endophilin b1 and Serpin b1a:

Full length murine cDNA's of Endophilin b1 and Serpin b1a were cloned into pENTR-D/Topo entry vector by setting up a PCR reaction using specific primers followed by Topo cloning reactions as per the manufacturer's instructions. Plasmid DNA was transformed into *E.coli* cells and selected on Kanamycin plates. The positive clones were then sequence confirmed.

The positive clones were then used for LR recombination reaction with Gateway Destination vectors i.e. pDEST 26, pDEST 27, pDEST Cmyc, pDEST GAD and selected on Ampicillin plates. The final positive clones were confirmed by sequencing and western blot.

3.3.2 Yeast Reverse Transformation:

The approach used here was similar to yeast transformation described previously. Here Full length pGBKT7-mHAX1 and pDESTGAD-mBif-1 were used for Yeast reverse transformation. pGBKT7-P53 and pGADT7-SV40 large T-antigen were used as positive controls. The transformed Yeast AH109 cells were plated on **SD Δ AHLT** plates and incubated for 3 to 5 days at 30^{o}C. Only in clones where both the proteins interact the reporter gene synthesis is activated and the clones would be able to survive with the essential amino acids of Adenine, Histidine, Leucine and Tryptophan.

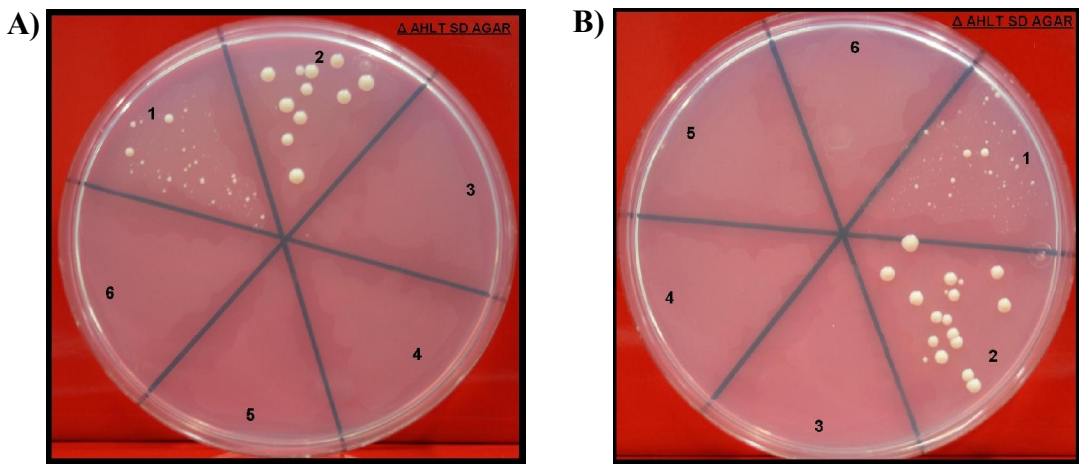


Fig 16: Yeast Reverse Transformation to confirm mHAX1-m Bif-1 and m HAX1- mSerpin b1a interactions. The Transformed cells were plated on **SD Δ AHLT** Agar plates and incubated for 3 to 5 days for the colonies to appear.

A) Lane 1: pGBKT7-mHAX1 and pDEST GAD-m Bif-1, Lane 2: pGBKT7-P53 and pGADT7-SV40 large T-antigen, Lane 3: pGBKT7-mHAX1 and pDEST GAD empty vector, lane 4: pDEST GAD-m Bif-1 and pGBKT7 only, Lane 5: pGBKT7-mHAX1, Lane 6: pDEST GAD-mBif-1.

B) Lane 1: pGBKT7-mHAX1 and pDEST GAD-m Serpin b1a, Lane 2: pGBKT7-P53 and pGADT7-SV40 large T-antigen, Lane 3: pGBKT7-mHAX1 and pDEST GAD empty vector, lane 4: pDEST GAD-m Serpin b1a and pGBKT7 only, Lane 5: pGBKT7-mHAX1, Lane 6: pDEST GAD-m Serpin b1a. In this experiment Lane 2 pGBKT7-P53 and pGADT7-SV40 large T-antigen was used s a positive control and Lane 3-6 were negative controls. Thus Interaction of m HAX1 with m Bif-1 and mSerpin b1a was proved by Yeast reverse transformation.

3.3.3 HAX1 Interacts with Serpin b1a:

Full length Murine Serpin b1a was cloned into pDEST GST and pDEST Cmyc expression vectors by gateway cloning.

pDEST Cmyc-mSerpin b1a and pEGFPC1-mHAX1 were transfected in 293T cells and the cells were harvested in 2% CHAPS lysis buffer and subjected to Immunoprecipitation by Anti-GFP. The samples were loaded on a 12.5% SDS gel and analyzed by western blotting against Anti-Myc.

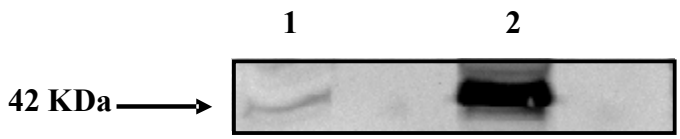


Fig 17: HAX1 interacts with Serpin b1a: GFP-mHAX1 and Cmyc-mSerpin b1a were transfected into 293T cells and the cell lysates were subjected to Immunoprecipitation by Anti-GFP. The samples were detected by western blot for C-Myc using Myc antibody. HAX1 was found to interact with Serpin b1a upon over-expression.

3.3.4 Immunoprecipitation of HAX1 and Bif-1:

To check for HAX1 and Bif-1 Interaction endogenously, 293T and Jurkat cells were cultured in complete DMEM and RPMI medium respectively and the cells were pelleted down by centrifugation at 900 rpm for 9 min. Total cell lysates were prepared in 2% CHAPS lysis buffer containing protease inhibitors and subjected to Immunoprecipitation with anti-Bif-1. Neat mouse serum was used a Negative control. The cell lysates were run on 12.5% SDS gel and western blot was done to detect for HAX1 using HAX1 antibody. This experiment confirms HAX1 - Bif-1 interaction at endogenous levels in 293T and Jurkat cell lines.

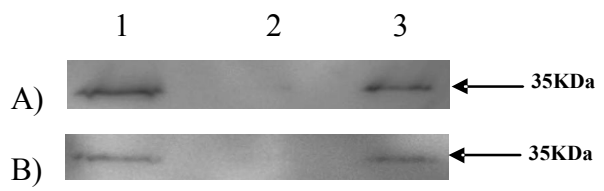


Fig 18: Immunoprecipitation in 293T and Jurkat cells with Bif-1 antibody.

A) IP was done in 293T cells Lane 1: total cell lysate, Lane 2: IP with Neat Mouse Serum, Lane 3: IP with Bif-1 Monoclonal antibody.

B) IP was done in Jurkat cells Lane 1: total cell lysate, Lane 2: IP with Neat Mouse Serum, Lane 3: IP with Bif-1 Monoclonal antibody. 10 µl of sample was loaded per lane and detected for HAX1 using HAX1 antibody. This confirms that HAX1 and Bif-1 interact endogenously in 293T and Jurkat cells.

3.3.5 Identification of HAX1 domain involved in the interaction with Bif-1:

In order to further confirm HAX1 interaction and to identify the region of HAX1 involved in interaction with Bif-1 GFP tagged deletion mutants of H.HAX1 were received from Dr Claudio Passananti. The details of the cloning strategy for these mutants are described previously.

293T cells were plated to 60% confluency in a 10 cm cell culture plate in complete DMEM medium and incubated overnight at 37^{o}C with 5% CO_2. The cells were transfected with 12.5µg of Plasmid DNA per plate using PEI protocol. The cells were then allowed to culture for a further 12 to 24 hrs for transfected gene to over express. GFP expression was checked in a fluorescent microscope and the cells were washed once with PBS and harvested in 1ml of 2% CHAPS lysis buffer containing protease inhibitors, and subjected to Immunoprecipitation with 5 µl of anti-GFP antibody. Samples were loaded on 12.5% SDS gels. Western blot was done using Bif-1 antibody (1:1000) in 5% Milk PBST overnight.

Endogenous Bif-1 was found to interact with Full length HAX1 and C-terminal (190-279 aa) deletion mutants, but not with N-terminal (1-190 aa) HAX1 deletion mutant. Further the interaction was confined to the C-terminal 179-234 amino acids of HAX1. This region of HAX1 was already reported to be involved in interacting with many other cellular proteins regulating many pathways. Also, only pEGFPC1 (empty vector) transfected were used as a negative control where IP was done with 5 µl of GFP antibody and the blot was detected for Bif-1 confirming that HAX1 interaction with Bif-1 is not mediated by the presence of GFP tag.

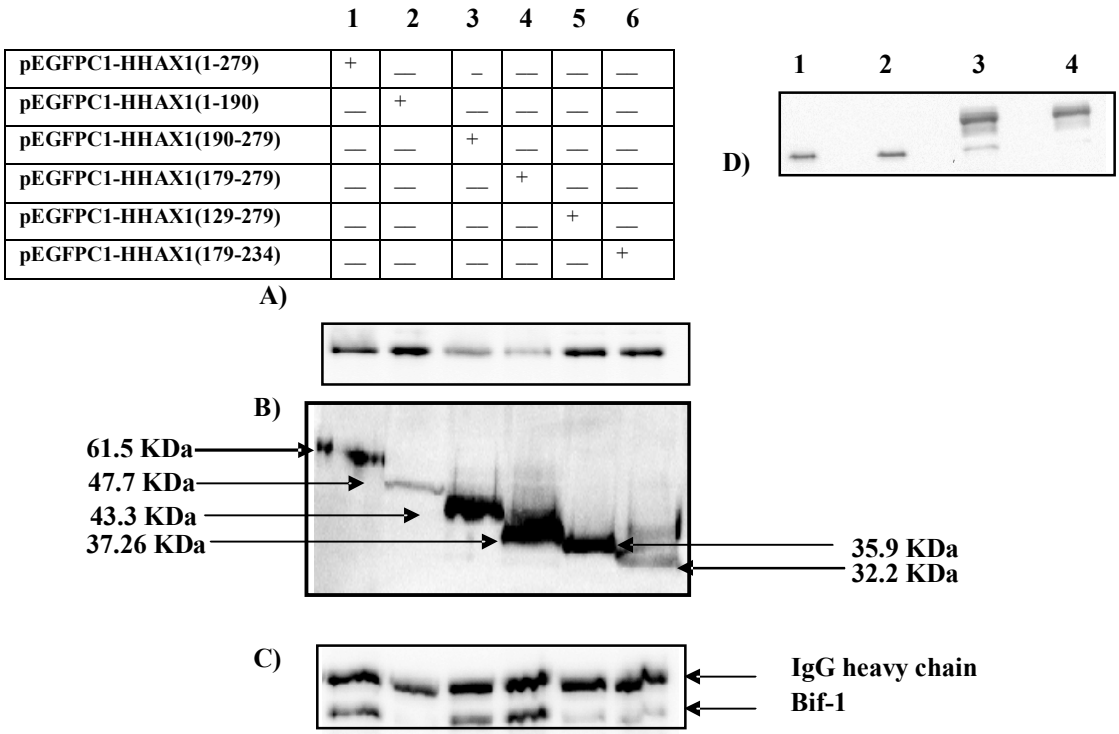


Fig 19: Immunoprecipitation to identify HAX1 domain involved in interaction with Bif-1.

A) Total cell lysates of the above samples after preclearing. 10µl of sample was loaded per lane and western blot was done to detect for Bif-1.

B) Total cell lysates of the above samples after preclearing. 10µl of sample was loaded per lane and western blot was done to detect for GFP. Lane 1 has HAX1 (1-279 aa) GFP Mol wt (61.5 KDa), Lane 2 has HAX1 (1-190 aa) GFP mol wt 47.40 KDa, Lane 3 has HAX1 (129-279 aa) GFP Mol wt 43.03 KDa, Lane 4 has HAX1 (180-279 aa) GFP Mol wt 37.26 KDa, Lane 5 has HAX1 (190-279 aa) GFP Mol wt 35.9 KDa and Lane 6 has HAX1 (179-234 aa) GFP Mol wt 32.2 KDa.

C) 12.5 µl of plasmid was transfected per reaction. IP was done using GFP antibody. 5 µl of antibody was used per reaction. Western blot was done to detect Bif-1. Lane 1: Full length GFP-HAX1 (1-279), lane 2: GFP-HAX1 (1-190). It was observed that N-terminal portion of HAX1 doesn't pull down Bif-1, Lane 3: GFP-HAX1 (190-279), Lane 4: GFP-HAX1 (179-279), Lane 5: GFP-HAX1 (129-279), Lane 6: GFP-HAX1 (179-234). This experiment proves that amino acids 179-234 of HAX1 in the C-terminal end are involved in Bif-1 interaction.

D) Control experiment using Empty Vector PEGFP-C1 was done to confirm that GFP alone doesn't interact with Bif-1.Lane 1: pEGFPC1-HHAX1 (1-279) transfected cell lysate after preclearing, Lane 2: Empty Vector pEGFPC1 alone transfected cell lysate after preclearing. Lane 3: Immunoprecipitation with GFP antibody in the lysate with pEGFPC1-HAX1 (1-279), Lane 4: Immunoprecipitation with GFP antibody in the lysate with pEGFPC1 empty vector. Western blot was done to detect for Bif-1 using Bif-1 antibody. This control experiment confirms that GFP alone doesn't interact with Bif-1.

3.3.6 Co-localization of HAX1 and Bif-1:

To determine the intracellular co-localization of HAX1 and Bif-1, HeLa cells were cultured on 12mm cover slips over night in complete DMEM medium. Primary antibody was added to the cells at a dilution of 1:50 in 1% BSA-PBST and incubated overnight at 4^{o}C in a moist chamber. Secondary antibodies Goat anti Mouse Alexa flour 633 and Goat anti rabbit Alexa flour 488 was added at a concentration of 1:500 and incubated at RT for 1 hr. The cells were thrice for 5 min each with PBST and20 µl of DAKO mounting media was added onto a glass slide and the cover slips were mounted on a glass slide with the cells facing the slide. The slides were incubated at 4^{o}C for 1 hr and the slides were sealed with nail polish and stored at -20^{o}C. The slides were observed under Confocal Laser scanning microscope with 63X oil immersion lens. Images were captured as described previously. The captured images were then analyzed for co-localization using WCIF ImageJ software. First the Background from the images was removed and the images were then merged and analyzed. The results of the analysis are as follows.

Hax1-Bif1 normal_Merging_04_ch01.tif and Hax1-Bif1normal_Merging_04_ch00.tif

Rr	R	ch1:ch2	M1	M2	Ch1 Thresh	Ch2 Thresh
0.772	0.836	1.003	0.999	0.997	0; 0	0; 0

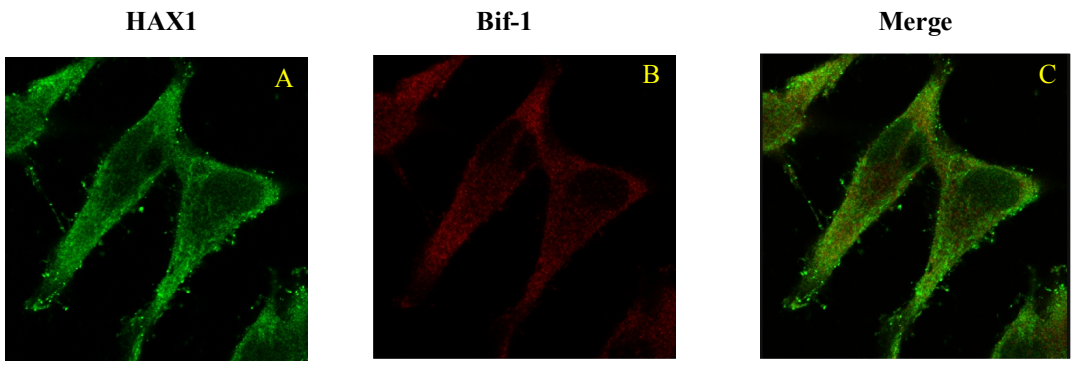


Fig 20: Confocal Laser Scanning Microscopic Analysis to determine HAX1 and Bif-1 Co-localization in HeLa cells.

A) HAX1 was detected using HAX1 antibody at a Conc. of 1: 50 and sec Ab used was Goat anti Rabbit Alexa flour 488,

B) Bif-1 was detected using Bif-1 antibody at a Conc. of 1: 50 and sec Ab used was Goat anti Mouse Alexa flour 488,

C) Merge. Images were analyzed using WCIF Image J software. The analysis of these images with WCIF image J indicates there is a colocalization of HAX1 and Bif-1.

3.3.7 Functional Characterization of HAX1 and Bif-1 interaction.

Bif-1 was reported to interact with Beclin1 through UVRAG and regulate EBSS starvation induced autophagy. In order to check the role of HAX1 in this Bif-1-UVRAG-Beclin1-PI(3KC3 complex in the initiation of autophagosome formation, 293T cells were transfected with pEGFPC1-H HAX1 (1-279) and cultured for 12 hrs. The cells were washed once with PBS and were treated with a) EBSS starvation for 4 hrs, b) 100 nm Rapamycin for 4 hrs and C) 1μM staurosporin for 4 Hrs. The cells were washed once with PBS and harvested in 2% CHAPS buffer and subjected to Immunoprecipitation by GFP antibody. 25 μl of sample was loaded per lane on SDS page gels and subjected to western blot detection by Bif-1, Beclin-1; PI(3)KC3 antibodies. It was observed that HAX1 interacts with Bif-1 in all the above mentioned conditions of apoptosis and autophagy. Also, HAX1 pulls down Beclin-1 and PI(3)KC3 indicating that HAX1 may have a possible role in the regulation of autophagy.

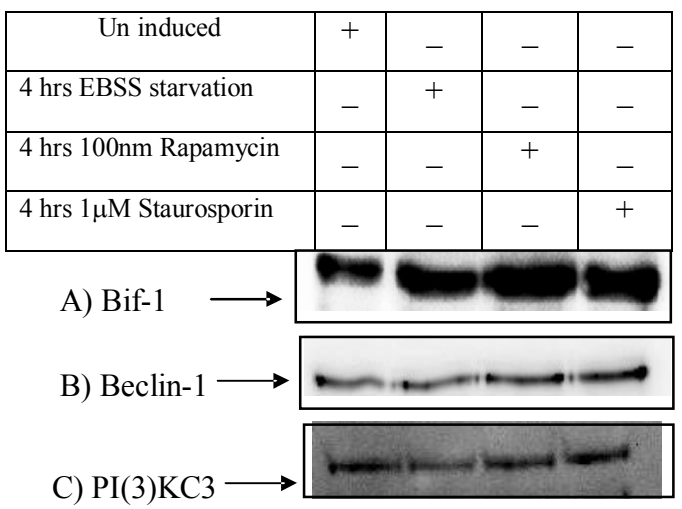


Fig 21: Functional Characterization of HAX1 and Bif-1 interaction.

Full length GFP tagged H HAX1 (pEGFPC1-HHAX1 1-279) was transfected in 293T cells in 10 cm plates and cultured for 12 hrs and the cells were subjected to Immunoprecipitation after inducing starvation for 4 hrs, treatment with 100 nm Rapamycin and treatment with 10 μm Staurosporin for 4 hrs.10 μl of GFP antibody was used for Immunoprecipitation. 25 μl of sample was loaded per lane and run on SDS gels.

The blots were then detected for A) Bif-1, B) Beclin1 and C) PI(3)KC3. This indicates that HAX1 interacts with Beclin-1 complex and might have a possible role in the initiation of autophagosome formation.

3.3.8 Role of HAX1 in EBSS induced starvation:

In order to check the possible role played by HAX1 in EBSS induced starvation Immortalized Skin fibroblasts from two normal donors and three HAX1 deficient patients were cultured in Complete DMEM medium. The cells were then cultured in EBSS starvation medium for 4 hrs and 8 hrs. Cells cultured in complete medium with out starvation were used as controls. Cells were harvested in hypertonic buffer and the protein concentrations were estimated by Bradford. 10 µg of protein was loaded per lane and western blot was performed to detect for Bif-1 and Phospho Thyrosine. β-actin was used as a loading control.

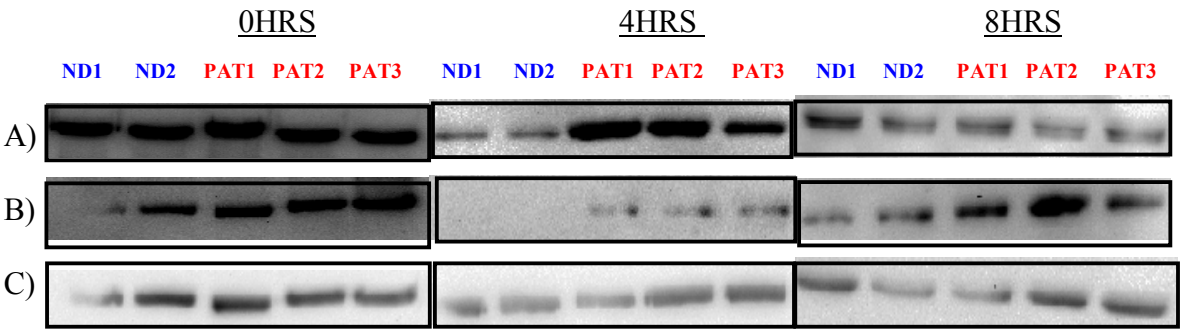


Fig 22: EBSS starvation of Normal Donor (ND) and HAX1-Deficient Patient (PAT) fibroblasts for 0Hrs, 4Hrs and 8 Hrs.
10 µg of protein was loaded per lane on 15% SDS page gels

A) Detected for Bif-1. The levels of Bif-1 were more in patients than compared to Normal donors after 4 hrs EBSS starvation.
B) Detected for Phospho Thyrosine. The levels of Phospho Thyrosine was more in patient fibroblasts when compared to normal donor controls at time points 4 and 8 hrs.
C) β-actin was used as a Loading control.
This indicates that there is an increased Phosphorylation of Bif-1 in HAX1 deficient patient fibroblasts than compared to Normal donor fibroblasts upon EBSS induced starvation.

These results suggest that HAX1 has a role in the phosphorylation of Bif-1, thus has a possible functional role in the induction of EBSS starvation induced autophagy.

3.3.9: Loss of HAX1 leads to Autophagic cell death after Nutrient starvation

Cell viability was tested using MTT cell proliferation Kit. Fibroblasts from Normal Donor and HAX1 deficient patients were seeded (5 X10^3) in triplicates in a 96 well tissue culture flat bottomed plate and incubated overnight at 37^{o}C. The cells were washed with PBS and then starved in 100 µl of EBSS starvation medium for 0Hrs, 4Hrs and 8Hrs

respectively with or with out pretreatment either with DMSO or 1µm ABT-737 and 10 mM 3-methyl adenine for 45 min.

10 µl of MTT labeling reagent was added per sample and incubated at 37°C for 4 hrs. 100 µl of solubilization solution was then added per sample and incubated at 37°C overnight. The samples were mixed well using a multi channel pipette. Measured the spectrophotometrical absorbance of the samples using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 550 and the reference wavelength used was 690 nm. The corrected value of A550nm-A690nm was used for statistical analysis.

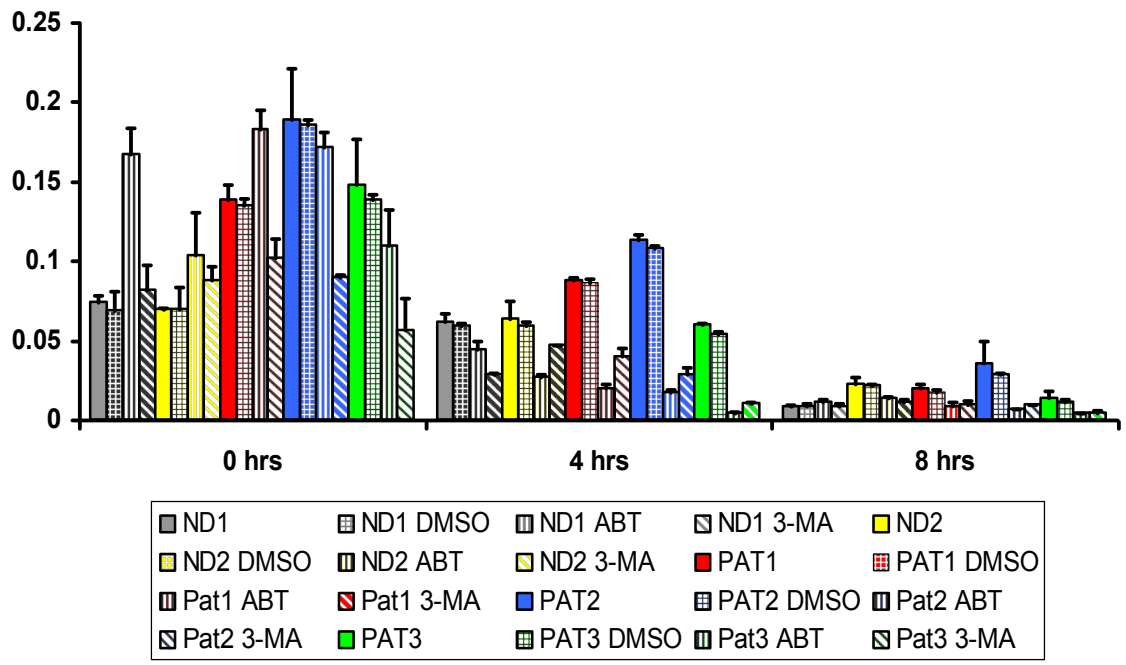


Fig 23: MTT assay to check for cell proliferation after EBSS starvation induced autophagy: Fibroblasts from two Normal donors and three patients were cultured in a 96 well plate in 100 µl of EBSS for 0 hrs, 4 hrs and 8 hrs respectively with and without pretreatment. Cells were pretreated with DMSO or 1µm ABT-737 or 10mM 3-Methyl adenine for 45 min and analyzed.

ND indicates Normal donor and PAT indicates patient fibroblasts. Two Normal donor and Three HAX1 deficient patient fibroblasts were used for the experiment.

It was observed that HAX1 deficient patient fibroblasts have higher cell viability when compared to Normal donor fibroblasts and upon Pretreatment with ABT-737 followed by EBSS starvation the loss of cell viability in HAX1 deficient patient fibroblasts was more compared to Normal donor fibroblasts. Upon pretreatment with 3-Methyl adenine the loss of cell viability was reduced to certain extent indicating that HAX1 deficient fibroblasts are more susceptible to Cell death by EBSS starvation than Normal donor Fibroblasts.

It was observed that HAX1 deficient Fibroblasts show more cell viability at 0 hrs when compared to Normal donor fibroblasts. Pretreatment with ABT-737 a BH3 mimicking Compound increases cell viability in normal donor fibroblasts but has no effect on HAX1 Deficient fibroblasts. There is a marked decrease in cell viability in HAX1 deficient fibroblasts at 4 Hrs EBSS starvation after ABT-737 pretreatment when compared to Normal donor fibroblasts. Pretreatment with 3-Methyl adenine a Beclin-1 complex Inhibitor helps to restore cell viability to a small extent. At 8 hrs EBSS starvation both Normal donor and HAX1 deficient fibroblasts were found to loose their viability indicating that most of the cells were dead.

3.3.10 Cell death by starvation is independent of Apoptosis.

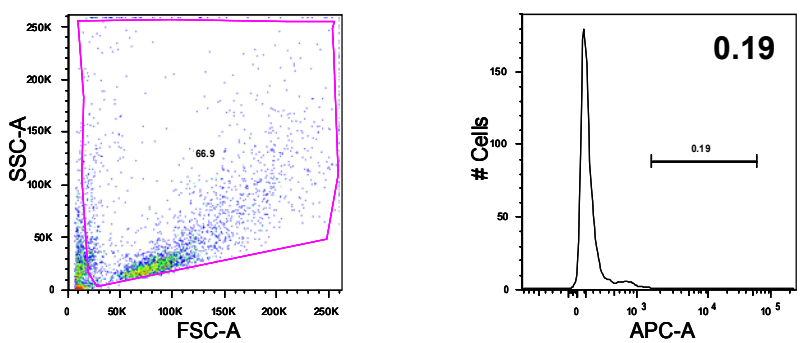
75,000 Fibroblasts from Normal donor and HAX1 deficient fibroblasts were seeded in a 6 well tissue culture plate and cultured over night in complete medium. The cells were then washed once with PBS and then cultured in 1 ml of EBSS medium for 0 hrs, 4 hrs and 8 hrs respectively. The cells were then stained with Annexin-V (APC) antibody and analyzed by FACS.

Fibroblasts treated with 10µM Camptothecin for 8 hrs and 48 hrs were used as a positive control. Cells cultured in complete medium and not stained for Annexin V were used as a negative control. In this experiment it was observed that starvation of Fibroblasts in EBSS medium does not lead to apoptosis as seen by Annexin-V staining.

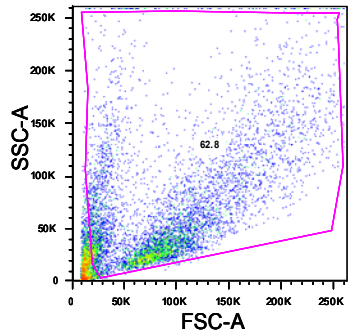
A)

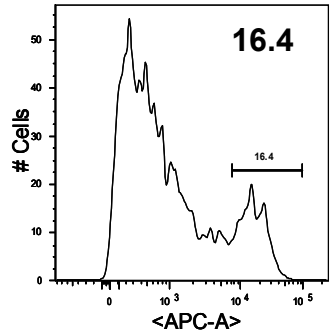
Control Staining

Unstained

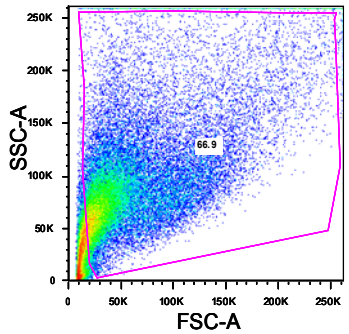


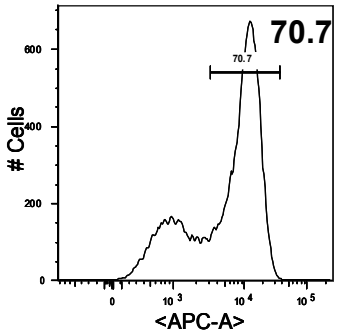
8 Hrs Camptothecin





48 Hrs Camptothecin

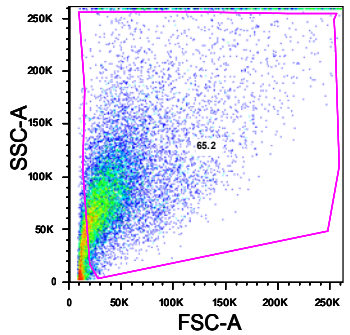


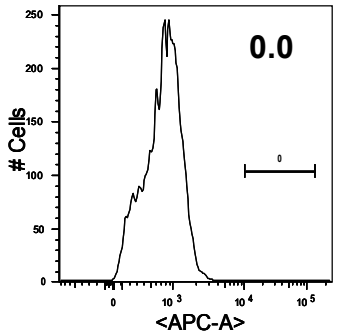


B)

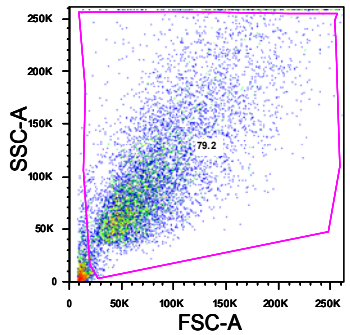
0 Hrs EBSS

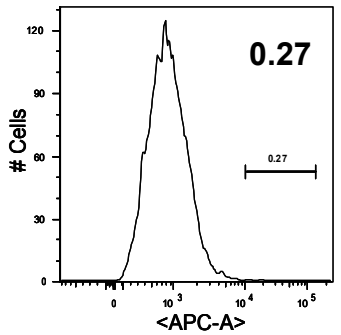
ND1



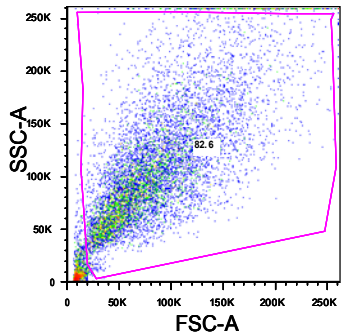


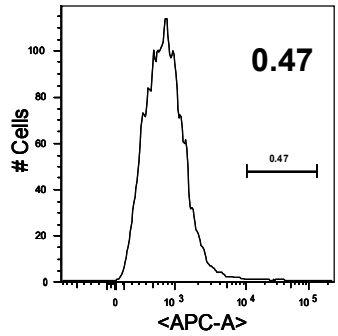
ND2



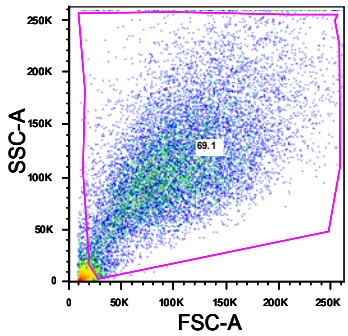


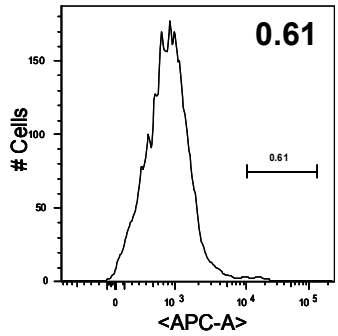
PAT 1





PAT 2





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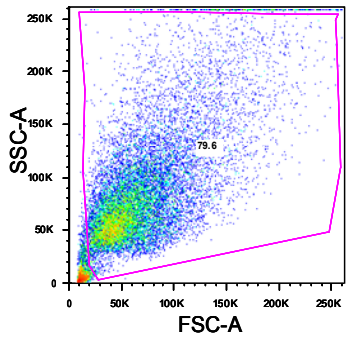
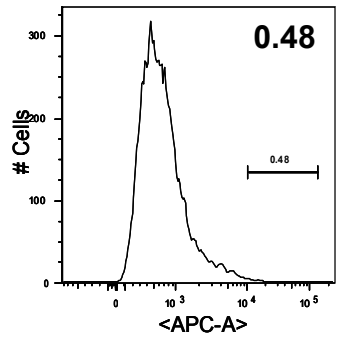
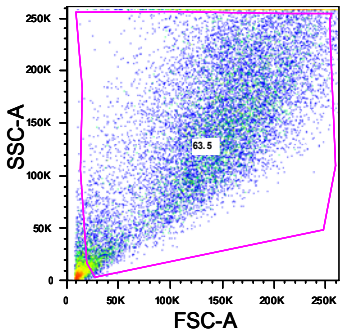


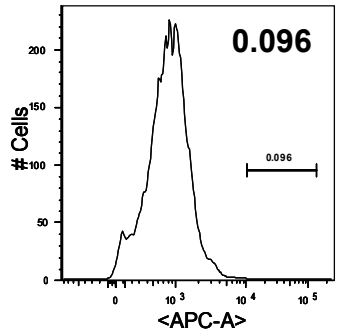


C)

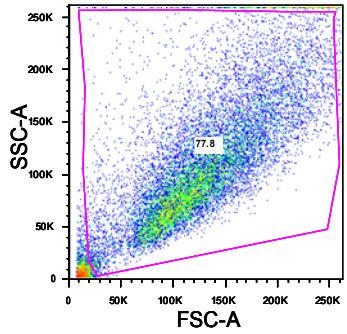
4 Hrs EBSS

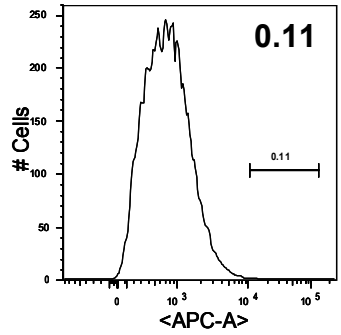
ND1



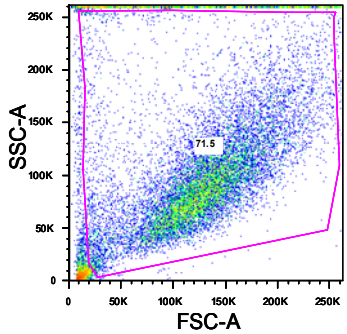


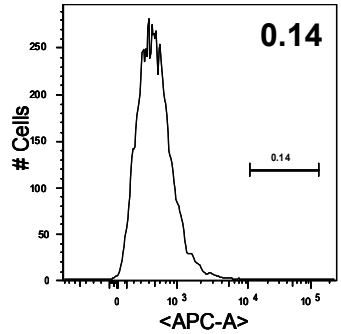
ND2



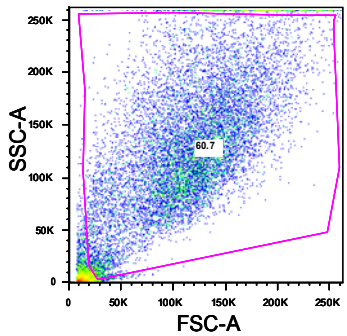


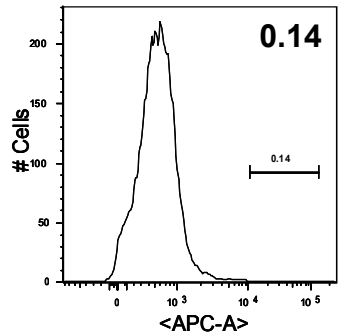
PAT 1





PAT 2





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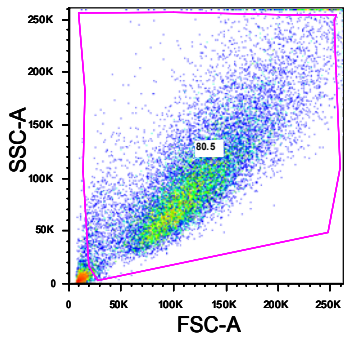
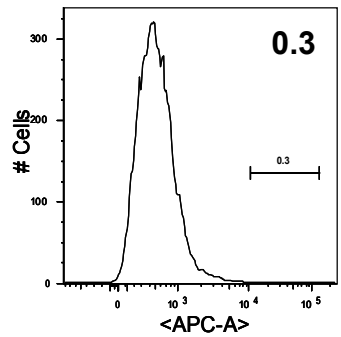
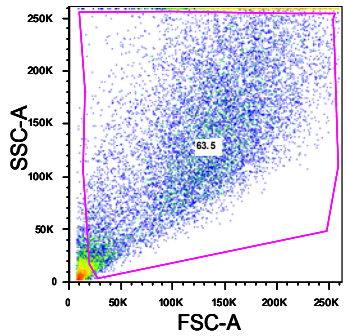


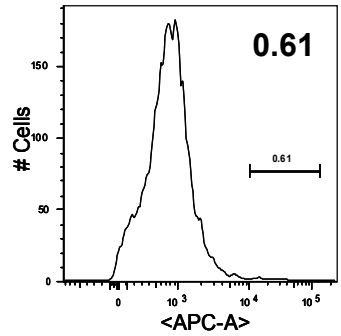


D)

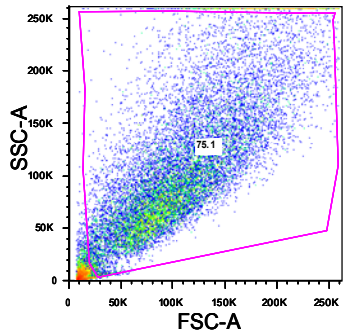
8 Hrs EBSS

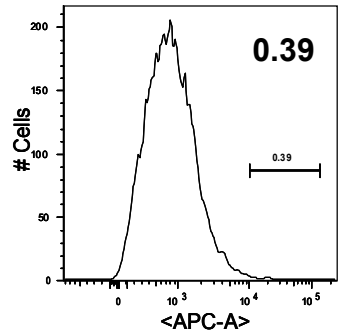
ND1





ND2





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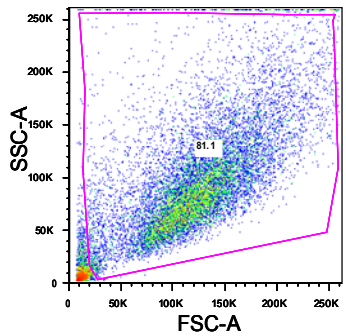
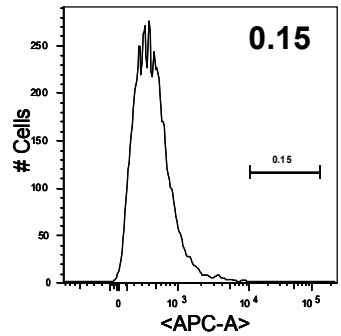
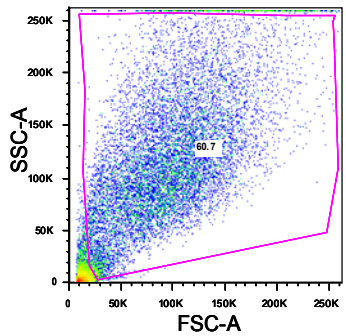
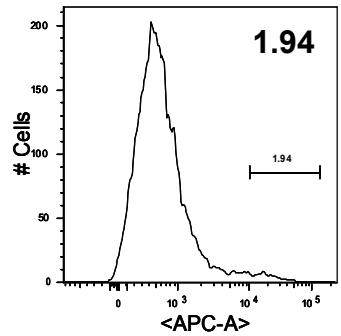




PAT 2





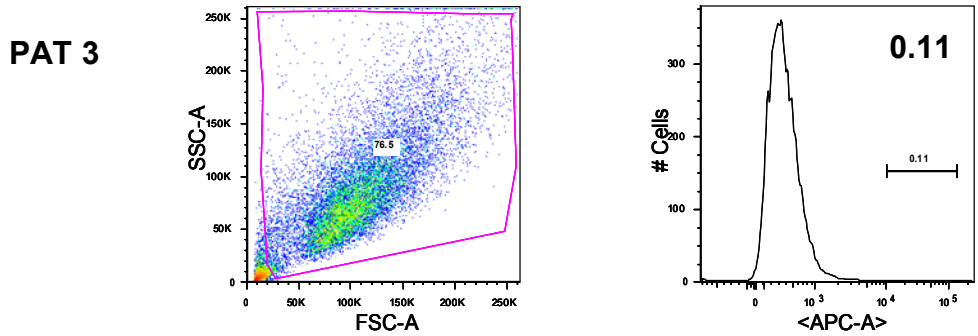


Fig 24: Cell death by Starvation is independent of Apoptosis

A) Cells which are untreated and Unstained, Cell treated for 8 hrs and 48 hrs respectively with 10µm Camptothecin were used as controls and stained for Annexin V (APC).

B) Normal donor and HAX1 deficient fibroblasts were stained for Annexin V at 0 hrs EBSS starvation. The percentage of Annexin V positive cells were less than 1% across the samples.

C) Normal donor and HAX1 deficient patient Fibroblasts were starved for 4 Hrs in EBSS starvation medium and stained for Annexin V. The percentage of Annexin V positive cells was negligible.

D) Normal donor and HAX1 deficient patient fibroblasts were starved for 8 hrs in EBSS starvation medium and stained for Annexin V. The percentage of Annexin V positive cells was negligible.

3.3.11 Starvation by EBSS leads to Caspase Independent Cell death

Fibroblasts from Normal donor and HAX1 deficient patient fibroblasts were cultured in triplicates in a 96 well tissue culture plate. 5,000 cells were seeded per well and cultured overnight in complete DMEM medium, the cells were then washed once with PBS and cultured in 100µl of EBSS medium for 0 hrs, 4 hrs and 8 hrs respectively. The samples were then tested for Active Caspase 3 and Active Caspase 7 using Caspase-Glo® 3/7 assay kit from Promega. 100 µl of the assay reagent was added per sample and incubated at room temperature in Dark for 2 hrs. The samples were then analyzed for Luciferase activity using Veritas™ Microplate Luminometer. Cells treated for 48 hrs with 10 µm Camptothecin were used as a positive control and the blank reaction consists of caspase3/7 reagent and EBSS medium.

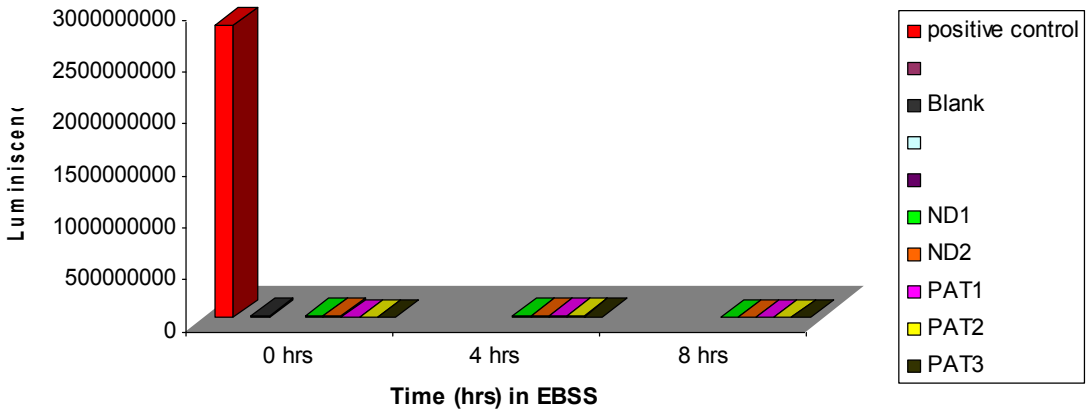


Fig 25: EBSS starvation leads to Caspase Independent Cell death.

Normal donor and HAX1 deficient patient fibroblasts were starved in EBSS medium for 0 hrs, 4 hrs and 8 hrs respectively and analyzed for Active Caspase 3 and 7 using Caspase Glo3/7 assay kit from Promega. Cells were cultured in 100 µl of EBSS medium and then 100 µl of the reaction reagent was added and incubated in Dark for 2 hrs at Room temperature. Cells treated with 10 µm Camptothecin for 48 hrs were used as a positive control while EBSS medium and Reaction reagent alone was used as Blank control. There was no active Caspase 3/7 observed upon starvation of cells in EBSS medium.

3.3.12 HAX1 is involved in Autophagy

To investigate the intracellular events that occur during nutrient deprivation, cells were examined by confocal laser scanning microscopy. Incubation in EBSS induced a large number of autophagic vacuoles in the cytoplasm of HAX1 deficient patient fibroblasts. as observed by the YFP–LC3 fusion protein punctuate foci formation, which is a well-characterized marker used to visualize autophagosomes, and represents the accumulation of a membrane- bound form of LC3 onto autophagic vacuoles. During autophagy, LC3 is processed from the cytosolic form (LC3-I) to the membrane-bound form (LC3-II). In order to test the role of HAX1 in autophagy a retroviral construct pQCXIX-YFP-LC3 was used to make retro virus and the viral supernatant was subsequently used to transduce fibroblasts from Normal donor and HAX1 deficient patient fibroblasts.

Viral titers were determined by transducing A549 cells with various dilutions of the thawed 36 hrs viral supernatant with 8µg of Polybrene per ml of culture. After 48 hrs the cells were harvested and tested by FACS for surrogate marker like GFP.

The viral titer for **pQCXIX-YFP LC3** is 2.91× 10 7 pfu/ml

3.3.13 Starvation of LC3 transduced Fibroblasts in EBSS medium.

One Normal Donor and One HAX1 deficient YFP-LC3 transduced fibroblasts were seeded on cover slips and cultured over night in complete DMEM medium. The cells were then starved in EBSS medium for 0 hrs and 4 hrs followed by fixation. The samples were analyzed using Laser scanning confocal microscopy.

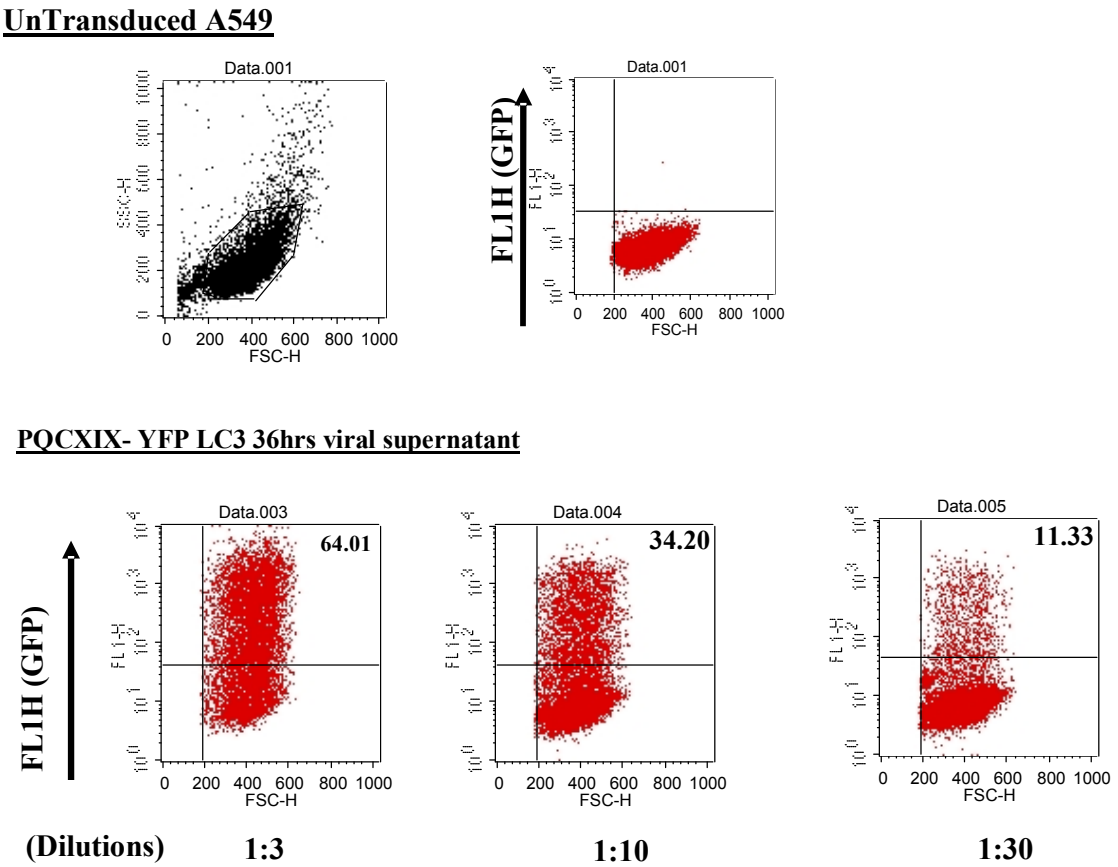
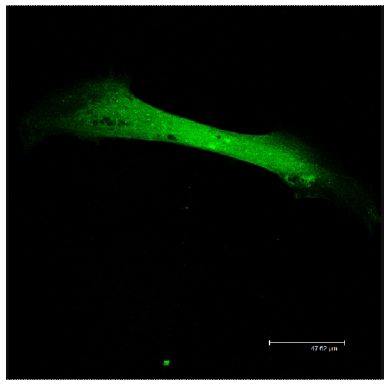
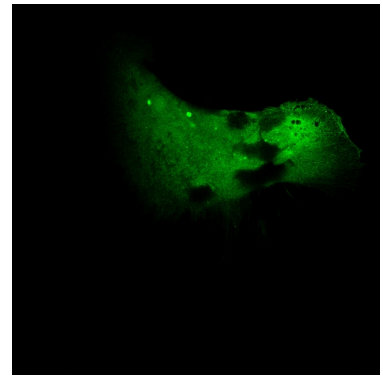


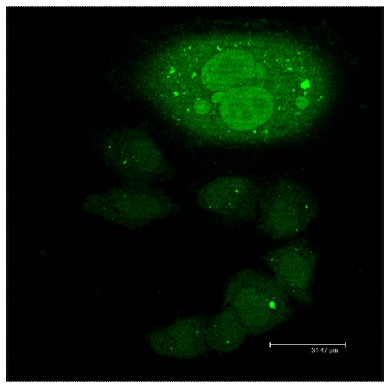
Fig 26: Determination of pQCXIX-YFP LC3 retroviral titer 1×10^{5} A549 cells were seeded in a 6 well tissue culture plate and cultured overnight. 3 dilutions of Virus was made (1:3, 1:10, 1:30) and used for transduction with 8 µg polybrene and the cells were harvested after 48hrs for FACS analysis for GFP expression. UnTransduced A549 cells were used as a control. The viral titer was calculated to 2.91× 10 7 pfu/ml.

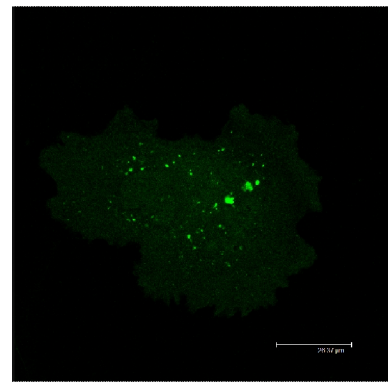
A) NORMAL DONOR FIBROBLASTS IN COMPLETE MEDIUM

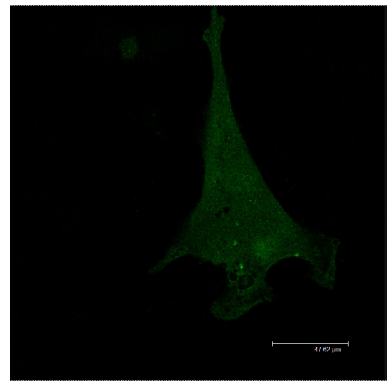




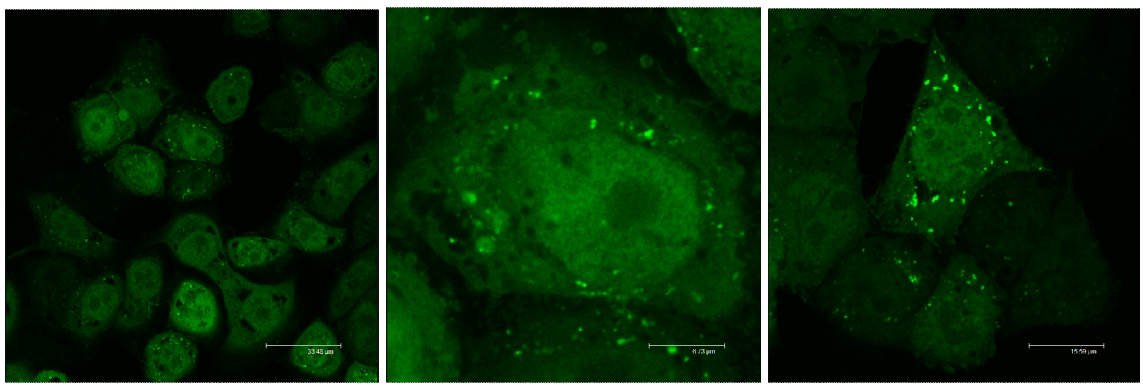
HAX1 DEFICIENT PATIENT FIBROBLASTS IN COMPLETE MEDIUM







B) **NORMAL DONOR FIBROBLASTS IN EBSS FOR 4 HRS**



HAX1 DEFICIENT PATIENT FIBROBLASTS IN EBSS FOR 4 HRS

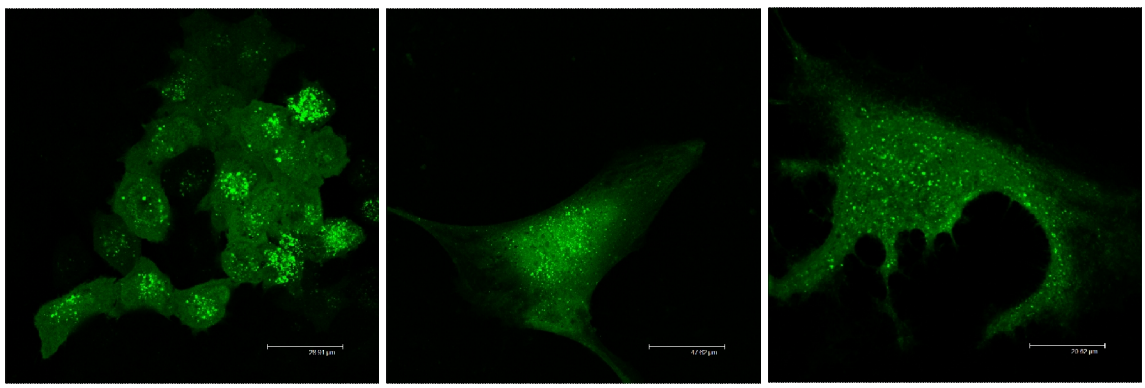


Fig 27: HAX1 is involved in Autophagy

pQCXIX-YFP LC3 transduced Normal donor and HAX1 deficient patient fibroblasts were cultured on cover slips and checked for YFP-LC3 punctuation using Confocal Laser Scanning microscopy.

A) Normal donor and HAX1 deficient patient fibroblasts were cultured in complete DMEM medium and analyzed for LC3 punctuation. The no of Punctures observed in HAX1 deficient fibroblasts is more than normal donor even when cultured in complete medium.

B) Normal Donor and HAX1 deficient patient fibroblasts were cultured in EBSS starvation medium for 4 hrs and analyzed for LC3 punctuation. The no of YFP-LC3 punctures were significantly more in HAX1 deficient patient fibroblasts than Normal donor indicating that Loss of HAX1 leads to Autophagic cell death upon starvation.

4. Discussion

The Kostmann Gene HAX1 is a mitochondria targeted protein with putative BH1 and BH2 domains. Although it has no BH3 domain it has some similarity to Nip3 a proapoptotic mitochondrial protein. Indeed, previous studies in various model systems have demonstrated that HAX1 possesses anti-apoptotic properties, and may suppress cell death through an interaction with pro-apoptotic proteins. Recently it was discovered that HAX1 is critical in maintaining mitochondrial membrane potential and protecting myeloid cells from apoptosis in SCN patients. While the role of HAX1 in cell death would thus appear to be a regulatory one, previous investigations have not been able to clarify just how HAX1 inhibits cell death nor has a detailed mechanism been proposed

4.1 Yeast Two Hybrid Screen

To understand how HAX1 plays a regulatory role in maintaining homeostasis inside the cell and how it regulates various cell survival and cell death pathways we have performed a yeast two hybrid screen using mHAX1 as bait and mated it with 17 day mouse embryonic cDNA library. The colonies on **SD Δ AHLT** were analyzed for β-galactosidase activity by X-gal colony lift filter assay. Blue colonies from this assay were analyzed and their plasmids extracted, and electroporated into *E.coli* and subsequently sequenced and analyzed using BLAST programme. 23 proteins were found to be potential interacting partners of HAX1. Some of these proteins were reported to be involved in apoptosis, autophagy, endocytosis and other cellular mechanisms.

4.2 Bio-chemical Characterization of HAX1

4.2.1 over expression of HAX1 in E.coli

To further characterize and understand the biochemistry of HAX1, cDNA's of murine and human HAX1 were cloned into *E.coli* expression vectors with His and GST tags for easier purification. Although there was considerable amount of protein expression with 3' His tag most of the protein was found to accumulate in the insoluble fraction. Overexpression was not observed with 5' GST tag.

4.2.2 Over expression and Purification of HAX1 in *Dictyostelium discoideum*

In view of these difficulties, I attempted to circumvent bacterial expression systems and to express HAX1 in Slime mold ***Dictyostelium discoideum***. This eukaryotic single celled motile amoeba was used successfully for expression and purification of Myosin and myosin fused fragments. We cloned Human HAX1 cDNA's into the pDXA vectors with 8X histidine tag and a catalytic domain of Myosin at the 5' end to which HAX1 gene was fused. HAX1 transformed ***Dictyostelium*** ORF^{+} cells were checked for HAX1 expression by coomassie blue staining and confirmed by Western blot. These cells were cultured in large scale and were subjected to protein purification by affinity chromatography using Ni-NTA column. Although considerable amount of expression was observed, there was no significant binding of the protein to the resin, thus making it difficult for purification. This could be due to the non accessibility of the Histidine Tag to the Nickel matrix. It was also observed that there is degradation of HAX1 when purified further to remove contaminating proteins by Gel filtration.

Further analysis of HAX1 using Bio-informatics tools suggested that HAX1 protein might be unstable. To overcome this we might have to over express and co-purify it with one of its interacting proteins.

4.3 Novel Interacting partners of HAX1:

Among the 23 proteins fished out by the yeast two hybrid screen, SH3GLB1 or Endophilinb1 and Serpinb1a were of considerable interest because

4.3.1 HAX1 Interacts with Serpin b1a:

Serpins are a super family of ~ 45-Kda proteins with a highly conserved tertiary structure. Serpins regulate important intracellular and extracellular proteolytic events; including apoptosis, complement activation, fibrinolysis, and blood coagulation (Potempa, Korzus et al. 1994),(Silverman, Bird et al. 2001). The seven-exon ov-serpin, Human Monocyte Neutrophil Elastase Inhibitor (MNEI, SERPINB1), is one of the most efficient inhibitor of the Neutrophil granule proteases, which include Neutrophil elastase, proteinase-3 (PR3), and cathepsin G (CatG) (Remold-O'Donnell, Nixon et al. 1989),(Remold-O'Donnell, Chin et al. 1992),(Sugimori, Cooley et al. 1995).

HAX1 was found to interact with Serpin b1a when checked by Yeast Reverse transformation and subsequently by Co-Immunoprecipitation. Understanding of HAX1's interaction with serpinb1a might shed light in understanding the molecular mechanism of Kostmann syndrome.

4.3.2 Endophilin b1 an evolutionarily conserved cytoplasmic protein that contains a predicted Src Homology 3 (SH3) domain located near its C terminus but shares no significant homology with members of the Bcl-2 family was found to interact with BAX (Sandy M. Cuddeback 2001).Over expression of Bif-1 promotes BAX conformational change, caspase activation and apoptotic cell death in FL5.12 cells following interleukin-3 deprivation (Sandy M. Cuddeback 2001). Bif-1 plays a regulatory role in apoptotic activation of BAX and BAK and is also involved in the suppression of tumorigenesis. Bif-1 didn't interact with BAK directly but heterodimerized with BAX on mitochondria in intact cells and their interaction was enhanced by apoptosis induction and preceded the BAX and BAK conformational change(Yoshinori Takahashi 2005) The reasons for the enhanced activation of BAX by Bif-1 during apoptosis were not clearly defined. In this respect Hirohito etal (Hirohito Yamaguchi 2008) discovered that Src Directly phosphorylates Bif-1 and prevents its interaction with Bax and the initiation of Anoikis. Bif-1 forms a complex with beclin-1 through UVRAG to enhance PI(3)KC3 lipid kinase activity and induce autophagosome formation during nutrient starvation (Yoshinori Takahashi 2007). EBSS induced PI(3)KC3 activation was only partially suppressed by loss of Bif-1, whereas the autophagosome formation was greatly blocked in *Bif-1*-deficient cells (Yoshinori Takahashi 2007). Bif-1 interacts with Beclin 1, through UVRAG, at the isolation membrane or phagophore, and thus regulates vesicle nucleation by inducing membrane curvature through its N-BAR domain during autophagy.

4.3.3 Confirmation of HAX1 and Bif-1 interaction by Yeast Reverse Transformation

To confirm the interaction of HAX1 and Bif-1 full length cDNA of murine Bif-1 was cloned into Yeast expression vector pDESTGAD and a Reverse Transformation assay was performed by transforming Yeast AH109 cells with pDESTGAD-mBif-1 and pGBKT7-mHAX1. These clones were able to survive and grow on **SD Δ AHLT** agar plates confirming that HAX1 interacts with Bif-1.

4.3.4 HAX1 and Bif-1 interact with each other endogenously

Further confirmation of this interaction was done by Immunoprecipitation experiments performed on cell lysates of 293T and Jurkat cell lines. The cells were harvested in 2% CHAPS buffer and subjected to Immunoprecipitation by using Anti Bif-1. The samples were analyzed by western blotting for HAX1 confirming that HAX1 and Bif-1 interact with each other in 293T and Jurkat cells at an endogenous level.

4.3.5 Bif-1 binds to the Carboxy terminal region of HAX1

Experiments with deletion mutants of H HAX1 fused to GFP were performed to elucidate the region of HAX1 involved in its interaction with Bif-1. In these experiments five deletion mutants spanning the entire length of HAX1 protein and a full length clone were used to identify the Bif-1 binding region of HAX1. Bif-1 was found to bind to C-terminal region of HAX1. There was no Bif-1 binding with the first 190 amino acids of HAX1. Further Bif-1 binding region was confined to the amino acids 179 to 234 located at the Proline rich C-terminal portion of HAX1. The C-terminal region of HAX1 was reported to be involved in many Protein-protein interactions. Also, SH3 domain containing proteins were reported to be involved in binding to proline rich motifs (Solomaha, Szeto et al. 2005)

4.3.6 HAX1 Co-localizes with Bif-1

Colocalization of HAX1 and Bif-1 was analyzed using HeLa cells. These cells were stained for HAX1 and Bif-1 and the images were taken using Laser Scanning confocal microscope. Upon analysis of the Images with ImageJ software for Mander's coefficient, it was confirmed that a significant portion of HAX1 and Bif-1 co-localize at endogenous levels.

The above in vitro experiments in Yeast and In vivo experiments in 293T, Jurkat and HeLa cells confirm that HAX1 and Bif-1 bind to each other endogenously and the C-terminal 179 to 234 amino acids of HAX1 interacts with Bif-1.

4.3.7 Functional Characterization of HAX1-Bif-1 Interaction

Bif-1 was reported to form a complex with beclin-1 through UVRAG enhancing PI(3)KC3 lipid kinase activity and inducing autophagosome formation during nutrient starvation (Yoshinori Takahashi 2007). EBSS induced PI(3)KC3 activation was only partially suppressed by loss of Bif-1, whereas the autophagosome formation was greatly blocked in *Bif-1*-deficient cells (Yoshinori Takahashi 2007). Bif-1 interacts with Beclin 1, through UVRAG, at the isolation membrane or phagophore, and thus regulates vesicle nucleation by inducing membrane curvature through its N-BAR domain during autophagy.

In order to check the role of HAX1 and its interaction with Bif-1 we have induced Starvation by culturing Fibroblasts from Healthy controls and HAX1 deficient patients in EBSS medium for different time points. The cells were harvested and their protein concentration was quantified and subjected to western blot analysis against different proteins. It was observed that the protein levels of Bif-1 were upregulated after 4 hrs starvation in Patient cells compared to the healthy controls thus confirming that Bif-1 upregulation plays a role in the induction of autophagy upon nutrient starvation. The comparable levels of Bif-1 protein in patient cells and healthy controls can be explained by the fact that cell death was almost complete as seen by the results of MTT cell proliferation assay.

One significant finding of these experiments is the increased phosphorylation of tyrosine in patient cells than healthy controls upon induction of autophagy by EBSS starvation. Recent reports suggest that phosphorylation of Bif-1 by protein kinase c-Src on tyrosine 80 impairs its binding to Bax and represses apoptosis (Yamaguchi, Woods et al. 2008) Taken together these findings suggests that Phosphorylation and de-phosphorylation of bif-1 may play a major role in the homeostasis of the cell, deciding its fate towards Apoptosis, autophagy or cell survival.

4.3.8 Role of HAX1 in Autophagy dependent cell death

To determine whether HAX1 is involved in autophagy dependent cell death, fibroblasts from HAX1 deficient patients and Healthy controls were cultured in EBSS solution and analyzed for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. At time zero the tetrazolium salt reduction was more in patient cells compared

to the healthy controls. But upon induction of autophagy by EBSS starvation, there was a drastic downregulation in the MTT reduction in patient cells while there was no significant change in the healthy controls. Upon continuation of starvation up to 8 hrs both, healthy controls and the patient cells were found to be non viable leading to significant decrease in the reduction of MTT.

ABT-737, a BH3 mimicking compound is known to competitively bind to Bcl2, Bcl-XL and Mcl-1 releasing Beclin-1 and there by activating the autophagosome formation by PI(3)-KC(3) activity leading to autophagic cell death.

When the cells were pretreated with 1µm of ABT-737 for 45 min followed by starvation in EBSS medium, HAX1 deficient patient fibroblasts were found to have significant decrease in their cell viability compared to normal donor controls.

3-Methyl adenine is a known Beclin-1 - PI(3) kinase complex inhibitor. This compound binds to beclin-1 complex and inhibits the activation of PI (3) kinase and finally blocking the formation of autophagosome.

Pretreatment of HAX1 deficient patient fibroblasts and Healthy controls with 10 mM 3-Methyl adenine lead to restoration of cell viability to an extent when compared to ABT-737 pretreated cells.

4.3.9 Nutrient deprivation leads to Caspase independent cell death.

HAX1 deficient fibroblasts and Normal donor controls were cultured in EBSS starvation medium for 0 hrs, 4 hrs and 8 hrs respectively. The samples were then analyzed for Annexin-v by FACS staining.

When ever a cell undergoes apoptosis, Phosphatidylserine which is generally present on the cytoplasmic side of the cell membrane is externalized. This is recognized by Annexin-V.

In this experiment when the cells were deprived of nutrients by EBSS starvation there were no Annexin-V positive cells even after 8 hrs of starvation indicating that nutrient deprivation leads to cell death independent of Apoptosis.

There was no Active Caspase-3 and Active Caspase-7 observed when the cells were deprived of nutrients when analyzed with Active Caspase Glo 3/7 kit. The luminescence observed in cells after starvation is similar to or less than Blank control, while cells treated with 10 µm Camptothecin had increased luminescence.

The above experiments indicate that Nutrient deprivation leads to Caspase independent cell death.

4.3.10 LC3 Punctuation assay

The no of autophagosomes in YFP-LC3 transduced HAX1 deficient fibroblasts were found to be more than the Normal donor controls when cultured in complete DMEM medium and the difference is very striking when the fibroblasts were deprived of nutrients by culturing in EBSS medium for 4 hrs indicating that Loss of HAX1 leads to Autophagy related cell death upon Nutrient deprivation.

4.3.11 Conclusion

Taken together these findings indicate that HAX1 interacts with bif-1 endogenously and colocalizes to a large extent in the cytoplasm. Loss of HAX1 leads to upregulation of Bif-1 and its tyrosine phosphorylation during autophagy by EBSS starvation. Loss of HAX1 also leads to cell death by autophagy upon EBSS starvation.

5. Future prospective

HAX1, a ubiquitously expressed cytoplasmic, multi functional protein was reported to play a regulatory role in maintaining the homeostasis of the cell.

In our studies we have detected several interacting proteins of HAX1 which could shed light on the potential involvement of HAX1 in various cellular processes and human diseases. A number of experiments are planned to follow-up detected interactions, map their interaction sites in more detail, and reveal physiological significance of the interactions.

HAX1 bearing retroviruses were constructed to transduce the patient and HAX1 Knock out cells and check for the reversion of the phenotype.

Experiments are designed to specifically block apoptosis by using Pan-caspase inhibitors during starvation and understand the molecular mechanism by checking for Phosphorylation of Bcl-2, Bcl-Xl and Mcl-1 proteins.

Serpinb1a interaction with HAX1 would be studied further using deletion mutants of HAX1. Their Intracellular localization would be analyzed by confocal microscopy under various apoptotic conditions. The functional significance of HAX1-serpin b1a would be understood by checking for the cleavage of Serpin in Knock out and Patient Bone marrow PBMC's and Neutrophil cells.

6. REFERENCES

Al-Maghrebi, M., H. Brule, et al. (2002). "The 3' untranslated region of human vimentin mRNA interacts with protein complexes containing eEF-1gamma and HAX-1." Nucleic Acids Res **30**(23): 5017-5028.

Arico, S., A. Petiot, et al. (2001). "The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway." J Biol Chem **276**(38): 35243-35246.

Arsham, A. M. and T. P. Neufeld (2006). "Thinking globally and acting locally with TOR." Curr Opin Cell Biol **18**(6): 589-597.

Ashkenazi, A. and V. M. Dixit (1998). "Death receptors: signaling and modulation." Science **281**(5381): 1305-1308.

Atlashkin, V., V. Kreykenbohm, et al. (2003). "Deletion of the SNARE vti1b in mice results in the loss of a single SNARE partner, syntaxin 8." Mol Cell Biol **23**(15): 5198-5207.

Baehrecke, E. H. (2003). "Autophagic programmed cell death in Drosophila." Cell Death Differ **10**(9): 940-945.

Bhaskar, P. T. and N. Hay (2007). "The two TORCs and Akt." Dev Cell **12**(4): 487-502.

Bishop, J. M., and Weinberg, R.A., eds. (1996). "Molecular Oncology " New York: Scientific American, Inc.

Buttner, S., T. Eisenberg, et al. (2006). "Why yeast cells can undergo apoptosis: death in times of peace, love, and war." J Cell Biol **175**(4): 521-525.

Carlsson, G., A. A. Aprikyan, et al. (2004). "Kostmann syndrome: severe congenital neutropenia associated with defective expression of Bcl-2, constitutive mitochondrial release of cytochrome c, and excessive apoptosis of myeloid progenitor cells." Blood **103**(9): 3355-3361.

Carlsson, G. and A. Fasth (2001). "Infantile genetic agranulocytosis, morbus Kostmann: presentation of six cases from the original "Kostmann family" and a review." Acta Paediatr **90**(7): 757-764.

Carlsson, G., I. van't Hooft, et al. (2008). "Central nervous system involvement in severe congenital neutropenia: neurological and neuropsychological abnormalities associated with specific HAX1 mutations." J Intern Med **264**(4): 388-400.

Cecconi, F. and B. Levine (2008). "The role of autophagy in mammalian development: cell makeover rather than cell death." Dev Cell **15**(3): 344-357.

Chao, J. R., E. Parganas, et al. (2008). "Hax1-mediated processing of HtrA2 by Parl allows survival of lymphocytes and neurons." Nature **452**(7183): 98-102.

Cho, K., L. Adamson, et al. (2002). "Identification of truncated form of mouse HAX-1s gene (HAX-1xs) and characterization of its expression in small intestine and thymus of mice after burn injury." Shock **18**(3): 223-229.

Cilenti, L., M. M. Soundarapandian, et al. (2004). "Regulation of HAX-1 anti-apoptotic protein by Omi/HtrA2 protease during cell death." J Biol Chem **279**(48): 50295-50301.

Clarke, P. G. (1990). "Developmental cell death: morphological diversity and multiple mechanisms." Anat Embryol (Berl) **181**(3): 195-213.

Codogno, P. and A. J. Meijer (2005). "Autophagy and signaling: their role in cell survival and cell death." Cell Death Differ **12 Suppl 2**: 1509-1518.

Cookson, S. L. F. a. B. T. (2005). "Apoptosis, Pyroptosis, and Necrosis: Mechanistic Description of Dead and Dying Eukaryotic Cells." INFECTION AND IMMUNITY **73**(4): 1907–1916.

Crighton, D., S. Wilkinson, et al. (2006). "DRAM, a p53-induced modulator of autophagy, is critical for apoptosis." Cell **126**(1): 121-134.

Cuervo, A. M. (2003). "Autophagy and aging--when "all you can eat" is yourself." Sci Aging Knowledge Environ **2003**(36): pe25.

Danial, N. N. and S. J. Korsmeyer (2004). "Cell death: critical control points." Cell **116**(2): 205-219.

Djavaheri-Mergny, M., M. Amelotti, et al. (2006). "NF-kappaB activation represses tumor necrosis factor-alpha-induced autophagy." J Biol Chem **281**(41): 30373-30382.

Dobreva, I., A. Fielding, et al. (2008). "Mapping the integrin-linked kinase interactome using SILAC." J Proteome Res **7**(4): 1740-1749.

Dorn, B. R., W. A. Dunn, Jr., et al. (2002). "Bacterial interactions with the autophagic pathway." Cell Microbiol **4**(1): 1-10.

Dufva, M., M. Olsson, et al. (2001). "Epstein-Barr virus nuclear antigen 5 interacts with HAX-1, a possible component of the B-cell receptor signalling pathway." J Gen Virol **82**(Pt 7): 1581-1587.

Edinger, A. L. and C. B. Thompson (2003). "Defective autophagy leads to cancer." Cancer Cell **4**(6): 422-424.

Espert, L., M. Denizot, et al. (2006). "Autophagy is involved in T cell death after binding of HIV-1 envelope proteins to CXCR4." J Clin Invest **116**(8): 2161-2172.

Esselens, C., V. Oorschot, et al. (2004). "Presenilin 1 mediates the turnover of telencephalin in hippocampal neurons via an autophagic degradative pathway." J Cell Biol **166**(7): 1041-1054.

Fadeel, B. and E. Grzybowska (2009). "HAX-1: A multifunctional protein with emerging roles in human disease." Biochim Biophys Acta.

Fimia, G. M., A. Stoykova, et al. (2007). "Ambra1 regulates autophagy and development of the nervous system." Nature **447**(7148): 1121-1125.

Gallagher, A. R., A. Cedzich, et al. (2000). "The polycystic kidney disease protein PKD2 interacts with Hax-1, a protein associated with the actin cytoskeleton." Proc Natl Acad Sci U S A **97**(8): 4017-4022.

Galluzzi, L., M. C. Maiuri, et al. (2007). "Cell death modalities: classification and pathophysiological implications." Cell Death Differ **14**(7): 1237-1243.

Germeshausen, M., M. Grudzien, et al. (2008). "Novel HAX1 mutations in patients with severe congenital neutropenia reveal isoform-dependent genotype-phenotype associations." Blood **111**(10): 4954-4957.

Gozuacik, D. and A. Kimchi (2007). "Autophagy and cell death." Curr Top Dev Biol **78**: 217-245.

Green, D. R., R. A. Knight, et al. (2004). "Ten years of publication in cell death." Cell Death Differ **11**(1): 2-3.

Grzybowska, E. A., E. Sarnowska, et al. (2006). "Identification and expression analysis of alternative splice variants of the rat Hax-1 gene." Gene **371**(1): 84-92.

Gutierrez, M. G., D. B. Munafo, et al. (2004). "Rab7 is required for the normal progression of the autophagic pathway in mammalian cells." J Cell Sci **117**(Pt 13): 2687-2697.

Han, Y., Y. S. Chen, et al. (2006). "Overexpression of HAX-1 protects cardiac myocytes from apoptosis through caspase-9 inhibition." Circ Res **99**(4): 415-423.
Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.
Hara, T., K. Nakamura, et al. (2006). "Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice." Nature **441**(7095): 885-889.
Harding, T. M., K. A. Morano, et al. (1995). "Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway." J Cell Biol **131**(3): 591-602.
Hippe, A., M. Bylaite, et al. (2006). "Expression and tissue distribution of mouse Hax1." Gene **379**: 116-126.
Hirohito Yamaguchi, N. T. w., Jay F. Dorsey, Yoshinori Takahashi, Nicole R. Gjertsen, Tomothy yeatman, Jie Wu, and Hong-Gang Wang (2008). "Src Directly Phosphorylates Bif-1 and prevents its interaction with BAX and the initiation of Anoikis." The Journal of Biological chemistry **283**(27): 19112-19118.
Ishikawa, N., S. Okada, et al. (2008). "Neurodevelopmental abnormalities associated with severe congenital neutropenia due to the R86X mutation in the HAX1 gene." J Med Genet **45**(12): 802-807.
Jager, S., C. Bucci, et al. (2004). "Role for Rab7 in maturation of late autophagic vacuoles." J Cell Sci **117**(Pt 20): 4837-4848.
Jin, Z. and W. S. El-Deiry (2005). "Overview of cell death signaling pathways." Cancer Biol Ther **4**(2): 139-163.
Jones, J. M., P. Datta, et al. (2003). "Loss of Omi mitochondrial protease activity causes the neuromuscular disorder of mnd2 mutant mice." Nature **425**(6959): 721-727.
Kamada, Y., T. Funakoshi, et al. (2000). "Tor-mediated induction of autophagy via an Apg1 protein kinase complex." J Cell Biol **150**(6): 1507-1513.
Kasashima, K., E. Ohta, et al. (2006). "Mitochondrial functions and estrogen receptor-dependent nuclear translocation of pleiotropic human prohibitin 2." J Biol Chem **281**(47): 36401-36410.
Kawaguchi, Y., K. Nakajima, et al. (2000). "Interaction of Epstein-Barr virus nuclear antigen leader protein (EBNA-LP) with HS1-associated protein X-1: implication of cytoplasmic function of EBNA-LP." J Virol **74**(21): 10104-10111.
Kawaguchi, Y., E. Nishimagi, et al. (2006). "Intracellular IL-1alpha-binding proteins contribute to biological functions of endogenous IL-1alpha in systemic sclerosis fibroblasts." Proc Natl Acad Sci U S A **103**(39): 14501-14506.
Kerr, J. F. (1971). "Shrinkage necrosis: a distinct mode of cellular death." J Pathol **105**(1): 13-20.
Kerr, J. F., A. H. Wyllie, et al. (1972). "Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics." Br J Cancer **26**(4): 239-257.
Kihara, A., Y. Kabeya, et al. (2001). "Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network." EMBO Rep **2**(4): 330-335.
Klein, C., M. Grudzien, et al. (2007). "HAX1 deficiency causes autosomal recessive severe congenital neutropenia (Kostmann disease)." Nat Genet **39**(1): 86-92.
Klionsky, D. J. (2005). "The molecular machinery of autophagy: unanswered questions." J Cell Sci **118**(Pt 1): 7-18.
Klionsky, D. J. (2007). "Autophagy: from phenomenology to molecular understanding in less than a decade." Nat Rev Mol Cell Biol **8**(11): 931-937.
Klionsky, D. J., J. M. Cregg, et al. (2003). "A unified nomenclature for yeast autophagy-related genes." Dev Cell **5**(4): 539-545.

Komatsu, M., S. Waguri, et al. (2006). "Loss of autophagy in the central nervous system causes neurodegeneration in mice." Nature **441**(7095): 880-884.
Komatsu, M., S. Waguri, et al. (2005). "Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice." J Cell Biol **169**(3): 425-434.
Kroemer, G., W. S. El-Deiry, et al. (2005). "Classification of cell death: recommendations of the Nomenclature Committee on Cell Death." Cell Death Differ **12 Suppl 2**: 1463-1467.
Kunz, J. B., H. Schwarz, et al. (2004). "Determination of four sequential stages during microautophagy in vitro." J Biol Chem **279**(11): 9987-9996.
Kvam, E. and D. S. Goldfarb (2007). "Nucleus-vacuole junctions and piecemeal microautophagy of the nucleus in S. cerevisiae." Autophagy **3**(2): 85-92.
Lee, A. Y., Y. Lee, et al. (2008). "HS 1-associated protein X-1 is cleaved by caspase-3 during apoptosis." Mol Cells **25**(1): 86-90.
Lee, C. H., K. Inoki, et al. (2007). "mTOR pathway as a target in tissue hypertrophy." Annu Rev Pharmacol Toxicol **47**: 443-467.
Lees, D. M., I. R. Hart, et al. (2008). "Existence of multiple isoforms of HS1-associated protein X-1 in murine and human tissues." J Mol Biol **379**(4): 645-655.
Leist, M. and M. Jaattela (2001). "Four deaths and a funeral: from caspases to alternative mechanisms." Nat Rev Mol Cell Biol **2**(8): 589-598.
Levine, B. and D. J. Klionsky (2004). "Development by self-digestion: molecular mechanisms and biological functions of autophagy." Dev Cell **6**(4): 463-477.
Levine, B., S. Sinha, et al. (2008). "Bcl-2 family members: dual regulators of apoptosis and autophagy." Autophagy **4**(5): 600-606.
Levine, B. and J. Yuan (2005). "Autophagy in cell death: an innocent convict?" J Clin Invest **115**(10): 2679-2688.
Li, W. B., J. Feng, et al. (2009). "Induction of apoptosis by Hax-1 siRNA in melanoma cells." Cell Biol Int **33**(4): 548-554.
Liang, C., P. Feng, et al. (2006). "Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG." Nat Cell Biol **8**(7): 688-699.
Liang, X. H., S. Jackson, et al. (1999). "Induction of autophagy and inhibition of tumorigenesis by beclin 1." Nature **402**(6762): 672-676.
Liang, X. H., L. K. Kleeman, et al. (1998). "Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein." J Virol **72**(11): 8586-8596.
M. Fengsrud, M. L. S., A. Øverbye, P.O. Seglen (2004). "Structural aspects of mammalian autophagy." Autophagy, Landes Bioscience: 11-24.
Majeski, A. E. and J. F. Dice (2004). "Mechanisms of chaperone-mediated autophagy." Int J Biochem Cell Biol **36**(12): 2435-2444.
Majno, G. and I. Joris (1995). "Apoptosis, oncosis, and necrosis. An overview of cell death." Am J Pathol **146**(1): 3-15.
Martins, L. M., A. Morrison, et al. (2004). "Neuroprotective role of the Reaper-related serine protease HtrA2/Omi revealed by targeted deletion in mice." Mol Cell Biol **24**(22): 9848-9862.
Mathew, R., S. Kongara, et al. (2007). "Autophagy suppresses tumor progression by limiting chromosomal instability." Genes Dev **21**(11): 1367-1381.
Matsuda, G., K. Nakajima, et al. (2003). "Epstein-Barr virus (EBV) nuclear antigen leader protein (EBNA-LP) forms complexes with a cellular anti-apoptosis protein

Bcl-2 or its EBV counterpart BHRF1 through HS1-associated protein X-1." Microbiol Immunol **47**(1): 91-99.
Matsuura, A., M. Tsukada, et al. (1997). "Apg1p, a novel protein kinase required for the autophagic process in Saccharomyces cerevisiae." Gene **192**(2): 245-250.
Meier, P., A. Finch, et al. (2000). "Apoptosis in development." Nature **407**(6805): 796-801.
Mirmohammadsadegh, A., U. Tartler, et al. (2003). "HAX-1, identified by differential display reverse transcription polymerase chain reaction, is overexpressed in lesional psoriasis." J Invest Dermatol **120**(6): 1045-1051.
Mizushima, N. (2005). "The pleiotropic role of autophagy: from protein metabolism to bactericide." Cell Death Differ **12 Suppl 2**: 1535-1541.
Mizushima, N. and D. J. Klionsky (2007). "Protein turnover via autophagy: implications for metabolism." Annu Rev Nutr **27**: 19-40.
Mizushima, N., B. Levine, et al. (2008). "Autophagy fights disease through cellular self-digestion." Nature **451**(7182): 1069-1075.
Modem, S. and T. R. Reddy (2008). "An anti-apoptotic protein, Hax-1, inhibits the HIV-1 rev function by altering its sub-cellular localization." J Cell Physiol **214**(1): 14-19.
Morris, D. R. and A. P. Geballe (2000). "Upstream open reading frames as regulators of mRNA translation." Mol Cell Biol **20**(23): 8635-8642.
Morris, E. J., K. Evason, et al. (2000). "Misdirected vimentin messenger RNA alters cell morphology and motility." J Cell Sci **113 (Pt 13)**: 2433-2443.
Munafo, D. B. and M. I. Colombo (2002). "Induction of autophagy causes dramatic changes in the subcellular distribution of GFP-Rab24." Traffic **3**(7): 472-482.
Nara, A., N. Mizushima, et al. (2002). "SKD1 AAA ATPase-dependent endosomal transport is involved in autolysosome formation." Cell Struct Funct **27**(1): 29-37.
Noda, T. and Y. Ohsumi (1998). "Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast." J Biol Chem **273**(7): 3963-3966.
Ohsumi, Y. (2001). "Molecular dissection of autophagy: two ubiquitin-like systems." Nat Rev Mol Cell Biol **2**(3): 211-216.
Potempa, J., E. Korzus, et al. (1994). "The serpin superfamily of proteinase inhibitors: structure, function, and regulation." J Biol Chem **269**(23): 15957-15960.
Prindull, G. (1995). "Apoptosis in the embryo and tumorigenesis." Eur J Cancer **31A**(1): 116-123.
Qu, X., J. Yu, et al. (2003). "Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene." J Clin Invest **112**(12): 1809-1820.
Qu, X., Z. Zou, et al. (2007). "Autophagy gene-dependent clearance of apoptotic cells during embryonic development." Cell **128**(5): 931-946.
Radhika, V., D. Onesime, et al. (2004). "Galpha13 stimulates cell migration through cortactin-interacting protein Hax-1." J Biol Chem **279**(47): 49406-49413.
Ramsay, A. G., M. D. Keppler, et al. (2007). "HS1-associated protein X-1 regulates carcinoma cell migration and invasion via clathrin-mediated endocytosis of integrin alphavbeta6." Cancer Res **67**(11): 5275-5284.
Reggiori, F., T. Shintani, et al. (2005). "Atg9 cycles between mitochondria and the pre-autophagosomal structure in yeasts." Autophagy **1**(2): 101-109.
Remold-O'Donnell, E., J. Chin, et al. (1992). "Sequence and molecular characterization of human monocyte/neutrophil elastase inhibitor." Proc Natl Acad Sci U S A **89**(12): 5635-5639.

Remold-O'Donnell, E., J. C. Nixon, et al. (1989). "Elastase inhibitor. Characterization of the human elastase inhibitor molecule associated with monocytes, macrophages, and neutrophils." J Exp Med **169**(3): 1071-1086.

Sandra Díaz-Troya, M. E. P.-P., Francisco J. Florencio and José L. Crespo (2008). "The role of TOR in autophagy regulation from yeast to plants and mammals." Autophagy **4**(7): 851-865.

Sandy M. Cuddeback, H. y., Kiyoshi Komatsu,Toshiyuki Miyashita, Masao Yamada, Chun Wu, Sujay Singh and Hong-Gang Wang (2001). "Molecular cloning and Characterization of Bif-1." The Journal of Biological chemistry **276**(23): 20559-20565.

Sarbassov, D. D., S. M. Ali, et al. (2005). "Growing roles for the mTOR pathway." Curr Opin Cell Biol **17**(6): 596-603.

Sarnowska, E., E. A. Grzybowska, et al. (2007). "Hairpin structure within the 3'UTR of DNA polymerase beta mRNA acts as a post-transcriptional regulatory element and interacts with Hax-1." Nucleic Acids Res **35**(16): 5499-5510.

Savill, J. and V. Fadok (2000). "Corpse clearance defines the meaning of cell death." Nature **407**(6805): 784-788.

Schaffer, A. A. and C. Klein (2007). "Genetic heterogeneity in severe congenital neutropenia: how many aberrant pathways can kill a neutrophil?" Curr Opin Allergy Clin Immunol **7**(6): 481-494.

Scott, S. V., A. Hefner-Gravink, et al. (1996). "Cytoplasm-to-vacuole targeting and autophagy employ the same machinery to deliver proteins to the yeast vacuole." Proc Natl Acad Sci U S A **93**(22): 12304-12308.

Sharp, T. V., H. W. Wang, et al. (2002). "K15 protein of Kaposi's sarcoma-associated herpesvirus is latently expressed and binds to HAX-1, a protein with antiapoptotic function." J Virol **76**(2): 802-816.

Shi, Y. (2002). "Mechanisms of caspase activation and inhibition during apoptosis." Mol Cell **9**(3): 459-470.

Shimizu, S., T. Kanaseki, et al. (2004). "Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes." Nat Cell Biol **6**(12): 1221-1228.

Silverman, G. A., P. I. Bird, et al. (2001). "The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature." J Biol Chem **276**(36): 33293-33296.

Solomaha, E., F. L. Szeto, et al. (2005). "Kinetics of Src homology 3 domain association with the proline-rich domain of dynamins: specificity, occlusion, and the effects of phosphorylation." J Biol Chem **280**(24): 23147-23156.

Sophie Pattingre , L. E., Martine Biard-Piechaczyk , Patrice Codogno (2007). "Regulation of macroautophagy by mTOR and Beclin 1 complexes." Biochimie **90**: 313-323.

Stromhaug, P. E. and P. O. Seglen (1993). "Evidence for acidity of prelysosomal autophagic/endocytic vacuoles (amphisomes)." Biochem J **291 (Pt 1)**: 115-121.

Sugimori, T., J. Cooley, et al. (1995). "Inhibitory properties of recombinant human monocyte/neutrophil elastase inhibitor." Am J Respir Cell Mol Biol **13**(3): 314-322.

Suzuki, K. and Y. Ohsumi (2007). "Molecular machinery of autophagosome formation in yeast, Saccharomyces cerevisiae." FEBS Lett **581**(11): 2156-2161.

Suzuki, Y., C. Demoliere, et al. (1997). "HAX-1, a novel intracellular protein, localized on mitochondria, directly associates with HS1, a substrate of Src family tyrosine kinases." J Immunol **158**(6): 2736-2744.
Takahashi, Y., D. Coppola, et al. (2007). "Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis." Nat Cell Biol **9**(10): 1142-1151.
Tanida, I., T. Ueno, et al. (2004). "LC3 conjugation system in mammalian autophagy." Int J Biochem Cell Biol **36**(12): 2503-2518.
Thompson, C. B. (1995). "Apoptosis in the pathogenesis and treatment of disease." Science **267**(5203): 1456-1462.
Thumm, M., R. Egner, et al. (1994). "Isolation of autophagocytosis mutants of Saccharomyces cerevisiae." FEBS Lett **349**(2): 275-280.
Tsukada, M. and Y. Ohsumi (1993). "Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae." FEBS Lett **333**(1-2): 169-174.
Vafiadaki, E., D. A. Arvanitis, et al. (2009). "The anti-apoptotic protein HAX-1 interacts with SERCA2 and regulates its protein levels to promote cell survival." Mol Biol Cell **20**(1): 306-318.
Vafiadaki, E., D. Sanoudou, et al. (2007). "Phospholamban interacts with HAX-1, a mitochondrial protein with anti-apoptotic function." J Mol Biol **367**(1): 65-79.
Verhagen, A. M., J. Silke, et al. (2002). "HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins." J Biol Chem **277**(1): 445-454.
Vogelstein, B. and K. W. Kinzler (2004). "Cancer genes and the pathways they control." Nat Med **10**(8): 789-799.
Wullschleger, S., R. Loewith, et al. (2006). "TOR signaling in growth and metabolism." Cell **124**(3): 471-484.
Xie, Z. and D. J. Klionsky (2007). "Autophagosome formation: core machinery and adaptations." Nat Cell Biol **9**(10): 1102-1109.
Yamaguchi, H., N. T. Woods, et al. (2008). "SRC directly phosphorylates Bif-1 and prevents its interaction with Bax and the initiation of anoikis." J Biol Chem **283**(27): 19112-19118.
Yedavalli, V. S., H. M. Shih, et al. (2005). "Human immunodeficiency virus type 1 Vpr interacts with antiapoptotic mitochondrial protein HAX-1." J Virol **79**(21): 13735-13746.
Yorimitsu, T. and D. J. Klionsky (2005). "Autophagy: molecular machinery for self-eating." Cell Death Differ **12 Suppl 2**: 1542-1552.
Yoshinori Takahashi, D. C., Norimasa Matsushita, Hernani D. Cualing,Mei Sun, Yuya Sato, Chengyu Liang, Jae U. Jung, Jin Q. Cheng, James J. Mulé, W. Jack Pledger and Hong-Gang Wang (2007). "Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis." nature cell biology **9**(10): 1142-1151.
Yoshinori Takahashi, M. K., Hirohito Yamaguchi, Aslamuzzaman Kazi, Jie Wu, Said M Sebti, Richard J. Youle, Hong-Gang wang (2005). "Loss of Bif-1 suppresses BAX/BAK cnformational change and Mitochondrial Apoptosis." Molecular and cellular biology **25**(21): 9369-9382.
Young, A. R., E. Y. Chan, et al. (2006). "Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes." J Cell Sci **119**(Pt 18): 3888-3900.

Yu, L., A. Alva, et al. (2004). "Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8." Science **304**(5676): 1500-1502.
Yue, Z., S. Jin, et al. (2003). "Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor." Proc Natl Acad Sci U S A **100**(25): 15077-15082.

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I would like to dedicate this work at the Divine Lotus Feet of My GOD, GUIDE, GAURDIAN and GOAL Bhagawan Sri Sathya Sai Baba. Swami, you are the only one who loved me as I am and who believed in me more than myself. You are the "WIND BENEATH MY WINGS" and your inspiration has been the foremost motivating factor of my life.

Erklärung zur Dissertation

Hierdurch erkläre ich, dass die Dissertation „**Identification and Characterization of HAX1 interacting proteins“** selbständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogenen Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, 24.09.2009

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Name: