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Fas-Activated Serine Threonine Kinase 4 is a Mitochondrial Protein

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Abstract of the Thesis

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Mitochondria play an essential role in eukaryotic cells, supplying energy levels which vastly exceed those available to prokaryotes. They rely heavily on the import of nuclear encoded proteins for their function. The FAST family of proteins which are synthesized in the cytoplasm and imported into mitochondria are an example of this. The FAST family has not been well characterized, and next to nothing is known about several FAST family members. In the following I have reviewed what is known about each member of the FAST protein family including conserved domains, structures, including roles in cellular function, apoptosis and disease. Additionally, our lab has begun to characterize FASTKD4, one of the least studied FAST family members. We have determined that FASTKD4 is both a cytoplasmic and a mitochondrial protein, it does not reside in the outer mitochondrial membrane, its overexpression is not acutely toxic, and preliminary results suggest that it is not a member of any large complex in resting cells.

I dedicate this work to my family - your support has been my inspiration….

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List of Abbreviations

2XYT	2X Yeast Extract Tryptone
3FH	Epitope tag containing 3 Flag and 6 histidine residues
6X-His	Epitope tag with 6 histidine residues
ATAD3	ATPase family AAA Domain-containing protein 3
ATP	Adenosine triphosphate
BCL-XL	B-cell lymphoma-extra-large pro-survival protein
BH3	Bcl-2 Homology region 3
BL21 RIPL	The BL21-CodonPlus competent E. coli cells
BNGE	Blue Native Gel Electrophoresis
ChIP	Chromatin immunoprecipitation
cDNA	complementary DNA
CLIP	cross-linking and immunoprecipitation
ConA	carbohydrate-binding protein
COS-7	Cell line derived from kidney cells of the African Green Monkey
COX	Cytochrome oxidase
DIF-1	aka IRF-2BP2 - Interferon regulatory factor-1 binding protein 2
DMEM	Dulbecco's modified eagle medium
EDTA	Ethylene diamine tetra acetic acid
FAST	Fas activated serine/threonine kinase
FASTKD	Fas activated serine/threonine kinase
FCCP	Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
HeLa	Immortal cell line derived from tumor in patient Henrietta Lacks
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IAP	Inhibitor of apoptosis
iCLIP	individual-nucleotide resolution cross-linking and immunoprecip.
IPTG	Isopropyl-Beta-D-Thiogalactoside
kD	kilo Dalton
LMP1	Latent infection membrane protein 1
LRPPRC	Leucine-Rich Pentatricopeptide Repeat Cassette
LSFC	Leigh syndrome, French-Canadian
MRC	Mitochondrial Respiratory Complex
MPP	Mitochondrial processing peptidase
MS	Mannitol sucrose
MSH	Mannitol sucrose HEPES
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA

NF-κB	nuclear factor *kappa*-light-chain-enhancer of activated *B* cells
NRIF3	Nuclear receptor interacting factor 3
nt	nucleotide
OCR	Oxygen consumption rate
P150 Buffer	Buffer consisting of 250 mM Hepes pH 8, PMSF, leupeptin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonyl Fluoride
RAP	RNA binding domain abundant in apicomplexans
RNA	Ribonucleic acid
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
Ser28	Serine residue #28
siRNA	Small interfering RNA
SLIRP	Stem loop interacting RNA binding protein
SOC Medium	Super Optimal broth with Catabolite repression
TAP	Tandem affinity purification
TIA-1	3'UTR mRNA binding protein
TIM	Translocase of the inner mitochondrial membrane
TNF-α	Tumor necrosis factor-*alpha*
TOM	Translocase of the outer mitochondrial membrane
TX-100	Triton X-100
U2OS	Human osteosarcoma cells
UTR	Untranslated region
UV	Ultraviolet
VSEL	Very small embryonic-like
YFP	Yellow Fluorescent Protein

Acknowledgments

Introduction

Endosymbiotic Theory - the origin of mitochondria and chloroplasts

The seeds of the endosymbiotic theory were sown as early as 1883 when French botanist Andreas Schimper suggested that "green plants may owe their origin to the unification of a colourless organism with one uniformly tinged with chlorophyll" based on his observation chloroplasts replicate by fission and not de novo (Schimper, 1883).

In 1905 Russian botanist Konstantin Mereschowski lent support to this by proposing that 1. "Two different organisms can stably coexist and in doing so may even give rise to a new type of organism" 2. "Plastids arise through division of pre-existing plastids, which arose from pre-existing plastids, etc. This continuity serves as a strong argument that they are foreign bodies or symbionts." 3. "Chromatophores can grow and divide independently of the nucleus and do not behave at all like organs, rather like independent organisms and must therefore be regarded as such or as symbionts" (Mereschowski, 1905, 1999).

In his 1923 "The Mitochondria Problem," Ivan Wallin extended the endosymbiotic theory from chloroplasts to mitochondria. The 'problem' was that if mitochondria indeed had a 'bacterial conception' as would be the case in an endosymbiotic event, "why then do mitochondria not respond to staining techniques in the same manner as bacteria, or why do they not possess pathogenic effects seen in bacteria," To address this, Wallin proposed that the symbiotic event itself was the cause for the difference. He suggested that "if mitochondria are of bacterial nature, we would not expect them to exhibit the properties of hardy, free-living microorganisms, but rather the properties of such forms that have developed an absolute symbiotic relationship." "It must, further, be emphasized that some influence in the host cell produces a limitation of growth of the symbiotic organism..." Wallin (1923).

Research exploring the theory of endosymbiosis largely faded until 1959 when American researchers C. Ralph Stocking and Ernest M. Gifford demonstrated that chloroplasts contained and replicated their own DNA. Stocking and Gifford showed that in isolated chloroplasts radioactively labeled thymidine was incorporated into newly synthesized chloroplast DNA (Stocking, 1959).

In 1961 Hans Ris and R. N. Singh imaged thin sections of blue green algae with an electron microscope, comparing them with bacteria, plant and animal cells. Their images revealed that while the general structure of blue green algae is different and a bit simpler in organization that typical plant or animal cells, the ultra-structure of the cell is remarkably similar to that found in bacteria and Streptomyces.   Previous electron microscopic studies had reported that the blue green algal cell was divided with the cell into different regions or 'plasms', including chromatoplasm and centroplasm. However, Ris and Singh found that there were indeed no sharp boundaries dividing the cell into special regions "plasms." Instead they reported a number of cell components with characteristic ultra-structure. They wrote "To begin with, it should be emphasized that blue-green algae do not have either chloroplast or grana. They contain lamellae, which in their origin and structure are very similar to the lamellae of chloroplasts. Perhaps the entire cell could be compared to an intact chloroplast" (Ris and Singh, 1961).

"On the origin of Mitosing Cells' published in 1967 by Lynn Margulis effectively reopened interest in the Endosymbiotic theory and transformed the study of evolution. Previously, most arguments in support of the endosymbiotic theory had relied on structure observations and comparative anatomical features. Margulis' work was revolutionary because it incorporated microbiological data. She proposed that evidence for an endosymbiotic event is the very existence of the mitochondria itself. If a symbiont originated as a free-living cell it must

have once been able to replicate its own DNA on its own synthesizing machinery. "...Upon entry into a host, such a symbiont may then lose from none to all of its synthetic capabilities except the ability to replicate its own DNA and synthesize complementary mRNA from that DNA - the *sine qua non* of any organism. If any of these organelles originated as symbionts, their characteristic specific DNA must be present in the host at every stage of the host life cycle" Sagan (1967).

Serial Endosymbiosis vs. the Hydrogen Hypothesis

There are currently two hypotheses regarding the mechanism of the endosymbiotic event. The first, Serial Endosymbiosis, holds that chloroplasts and mitochondria are the results of incorporation and semi-integration of cyanobacteria and α-proteobacteria, respectively, into a host cell approximately 2 billion years ago (Taylor, 1974). The second strategy is known as the Hydrogen Hypothesis. It proposes that the host cell which acquired the mitochondria was a hydrogen dependent archaea, and that the future mitochondrion was a facultatively anaerobic eubacterium which produced hydrogen and carbon dioxide as byproducts of anaerobic respiration. A symbiotic relationship arose when the host became dependent on hydrogen (Martin and Muller, 1998). Modern examples can be found of methanogens clustering around hydrogenosomes within eukaryotic cells to give an indication of how an endosymbiosis may have occurred originally (de Graaf et al., 2009).

Interestingly, it has recently been shown via genetic analysis of the complete genome of a hydrogenosome parasite in the hindgut of a cockroach, that hydrogenosomes and mitochondria share a common α -proteobacterial ancestor. Evidence of lateral gene transfer suggests that the hydrogenosome was originally a mitochondrion that became a hydrogenosome secondarily (Akhmanova et al., 1998).

Gene transfer from mtDNA to the nucleus

Present day mammalian mtDNA is much smaller than the original α-proteobacterial symbiont bacteria DNA. According to genetic analysis, over the course of time since the endosymbiotic event, most of the original bacterial genes have been transferred to nuclear DNA (Timmis et al., 2004). As a result, present day mitochondria must import the majority of the proteins they need for biogenesis and function from the cytoplasm. Nuclear-encoded proteins required within the mitochondria are made on cytoplasmic ribosomes and imported into the mitochondria via transportation complexes such as TOM and TIM. For example, the formation of the mitochondrial respiratory complexes requires nuclear encoded proteins that complex with mtDNA encoded proteins inside the mitochondria to comprise the final functional product (Cheng and Ivessa, 2010).

Mammalian mtDNA is maternally inherited

Mitochondria and their contents, including multiple mtDNA molecules, are normally inherited exclusively maternally, passed down from mother to child in the egg. However, upon fertilization sperm has been found to contribute about 100 mitochondria to the fertilized egg. These mitochondria are normally promptly destroyed inside the developing embryo, having been marked with ubiquitin in the male reproductive tract. In very rare cases some paternal mitochondria escape destruction and paternal mtDNA remains in the fertilized egg resulting in a condition known as heteroplasmy in the offspring (Sutovsky et al., 1999).

Notably, the ubiquitin tagging and subsequent destruction of paternal mitochondria is species dependent. An interspecies cross was performed with sperm from wild gaurs introduced into domestic cow eggs. The wild gaur sperm mitochondria were shown to persist beyond the third embryonic division and were not ubiquitinated (Sutovsky et al., 1999).

Because mtDNA is maternally inherited, not recombined in the manner of nuclear DNA, and steadily accrues mutations, it can be useful in performing phylogenetic analyses. By comparing mtDNA sequences within and between species it is possible to build phylogenetic trees and determine relatedness. This has been done extensively for many organisms, including humans. In a landmark 1987 study, mtDNA sequences from 147 people, drawn from five geographic populations were analyzed by restriction mapping. Based on phylogenetic analysis, it was determined that all the mitochondrial DNAs in the study derived from one woman thought to have been alive 200,000 years ago in Africa (Cann et al., 1987).

mtDNA variability

The genetic code of nuclear DNA is so very nearly universal that most life on earth, with few exceptions, adheres to it. The few exceptions include ciliate protozoa, arctic algae and a parasitic flagellate that lives in the intestines of birds. The genetic code of mtDNA is far more variable, with 12 known mitochondrial genetic codes among various algae, yeast, mold, coelenterates, echinoderms and flatworms (Andrzej, 2012). The physical structure of mtDNA across species is highly variable. While human mtDNA is one small, circular, compact molecule, the mtDNA of plants and other species can differ widely. For example, in maize mitochondria, there are two linear mtDNA plasmids of different sizes (Weissinger et al., 1982). In at least thirteen yeast species and many species of plants mtDNA molecules are a combination of both linear and circular mtDNA (Backert, 1993) (Fukuhara et al., 1993).

Gene structure of mammalian mtDNA

Mammalian mtDNA is very compact and contains very few genes.   It is a circular double stranded molecule containing about 16,569 base pairs. One strand is known as the "heavy strand" and the other is the "light strand." The heavy strand, coding for 28 genes, is so named due to its higher content of guanine and thymidine and resultantly higher density. The light strand, coding 9 genes, has a higher content of nucleotide cytosine and is relatively lower in density (Attardi and Schatz, 1988).

Human mtDNA encodes 37 genes, the products of which are exclusively mitochondrial: 13 of these genes code for core components of the electron transport chain: 7 subunits of Complex I, Cytochrome b - subunit 3 of Complex III, Subunits I, II, and III of Complex IV, and 2 subunits of Complex V - also known as ATP synthase (Anderson et al., 1981). The remaining 24 mtDNA genes encode the 12S and 16S ribosomal RNA, components of the mitochondrial ribosome and 22 mitochondria-specific tRNAs (Attardi and Schatz, 1988). Everything else required for mitochondrial biogenesis and function must be imported from the cytosol.

mtDNA transcription and translation

mtDNA features heavy strand and light strand promoters that when initiated produce two long, polycystronic transcripts (Bogenhagen et al., 1984) (Montoya et al., 1982) (Chang and Clayton, 1984). The primary transcript of the heavy strand contains no introns but has tRNA sequences positioned between and adjacent to rRNA and/or mRNA sequences (Attardi and Schatz, 1988). The final products are generated by precise endonucleolytic cleavage which occurs, in most cases, immediately before and after a tRNA sequence in a process described in 1981 by Giuseppe Attardi and colleagues as the 'tRNA punctuation model' (Ojala et al., 1981). Transcription and processing of the light strand produces either small transcripts which become

primers for mtDNA replication (linking mtDNA transcription and replication) or one long

transcript which is cut into functional tRNA, rRNA, and mRNA molecules (Chang et al., 1987).

mtDNA encoded proteins are produced within the mitochondria by ribosomes which are

assembled within the mitochondria. Almost all reading frames contain no significant

untranslated flanking regions. Most reading frames have no complete termination codon and end

instead with either a T or TA following the last codon. In these cases, the termination codon

TAA is completed post transcriptionally by polyadenylation of the mRNAs (Montoya et al.,

1981).

Mitochondrial disease

Because mitochondria rely on both nuclear DNA encoded proteins and their own

mtDNA- encoded proteins for proper function, they are doubly susceptible to disease resulting

from genetic mutation.   That is to say, mitochondrial disease can result from either mutant

mtDNA coded products or mutant nuclear DNA encoded products destined for the mitochondria.

mtDNA is present in multiple copies in a single mitochondrion, for an average total of

1,000 - 10,000 mtDNA copies in each human somatic cell. Most cells are homoplasmic: They

contain only one mtDNA sequence, which is present in multiple copies. However, if a mutation

arises while replicating the mtDNA, heteroplasmy can occur. Even if the mutation which has

caused heteroplasmy is deleterious, it may not affect the cell adversely. Depending on the cell or

tissue type, not until the proportion of mutant mtDNA to normal mtDNA exceeds a threshold,

does mtDNA disease result (Johns, 1995).

Nuclear encoded mitochondrial proteins have a wide range of functions in the

mitochondria including forming the core of members of the electron transport chain,

metabolizing fats, steroid synthesis, regulation of apoptosis, mitochondrial ribosomal assembly

and the stabilization of mitochondrial mRNAs, to name just a few. LRPPRC is an example of a nuclear encoded mitochondrial protein whose function is currently under investigation. LRPPRC has been found to complex with SLIRP (a mitochondrial RNA binding protein) to suppress 3' exonucleolytic mRNA degradation and also to promote the polyadenylation of mitochondrial mRNAs. Deleterious mutations in LRPPRC have been associated with the French-Canadian type of Leigh syndrome or LSFC, which is usually fatal by the age of 5 or 6. Affliction with LSFC leads to degeneration of the brain as well as metabolic abnormalities resulting from a deficiency in cytochrome oxidase - Complex IV of the mitochondrial respiratory complex (Chujo et al., 2012).

Protein import into mitochondria

Because the proteome of the human mitochondria consists of about 1,500 different proteins, with only 13 of these being encoded by mitochondrial DNA, there is a complex and precise system for bringing proteins into the mitochondria (Taylor et al., 2003). Proteins destined for the mitochondria are synthesized as precursor proteins in the cytosol, guided to the mitochondria, and escorted to their proper final locations in one of several compartments: The outer or inner mitochondrial membranes, the inter-membrane space, or the matrix (Endo et al., 2003).

First, cytosolic chaperones escort mitochondrial precursor proteins to receptors located on the outer mitochondrial surface. The type of mitochondrial targeting signal a protein bears determines it final destination. There are two general types of mitochondrial targeting signals: The first is a positively charged N-terminal series of amino acids known as a presequence. The majority of proteins bearing such N-terminal sequences are destined for the mitochondrial matrix, with just a few being targeted to the inner membrane and inter-membrane space. The

presequence mitochondrial targeting signals interact with mitochondrial import receptors which direct the preproteins across both outer and inner membranes (Koehler et al., 1999). Once inside the mitochondria, the presequence is recognized by and removed by the mitochondrial processing peptidase MPP (Gakh et al., 2002). The second type of mitochondrial targeting signal is a somewhat variable internal sequence. Proteins that carry this internal sequence include all outer membrane proteins and many inter-membrane space and inner membrane proteins. They are synthesized without cleavable extensions, so that they have the same primary structure as the mature protein, but their conformation changes once inside the mitochondria (Koehler et al., 1999) (Wiedemann et al., 2004).

The FAST family of proteins

The FAST Family or Fas-Activated Serine-Threonine Family of Kinases is a relatively unstudied family of nuclear encoded proteins which are imported to the mitochondria. Six members of the protein family have been identified: FAST and FASTKD1-5. Each FAST family protein is between 515 - 847 amino acids long and contains a shared set of definitive features: A positively-charged, N-terminal mitochondrial targeting sequence, a RAP domain and two FAST kinase domains (Simarro et al., 2010).

The RAP domain, or RNA-binding domain abundant in Apicomplexans, is composed of about 60 amino acids and includes blocks of charged and aromatic residues. The domain contains three α-helices and six β-sheets and is thought to bind RNA during splicing reactions. The RAP domain is present in the catalytic domains of many restriction endonucleases such as: EcoRI, BamHI, and FokI (Lee, I. Hong, W., 2004).

Each FAST Kinase Domain, designated FAST_1 and FAST_2, contains leucine rich regions that are thought to promote protein-protein interactions. It must be noted that these

leucine rich domains are not of the leucine zipper type.   That there is very little conserved

sequence outside the two kinase domains and the RAP domain suggests an independent

evolution for each FAST family member (Simarro et al., 2010).

Each FAST family protein has a confirmed mitochondrial location. COS-7 cells were

transiently transfected with plasmids encoding fusion proteins consisting of each of the FASTKD

members fused at their carboxyl termini to yellow fluorescent protein (YFP). The cells were

then stained with human anti-mitochondrial antigen M2 and analyzed by immunofluorescence.

The intracellular localization of FASTKD1-YFP- FASTKD5-YFP overlapped perfectly with

mitochondria labeled with human antibody against mitochondrial antigen M2 (Simarro et al.,

2010). Similarly, in 2004 it was demonstrated that FAST has mitochondrial localization. HeLa

cells stained with antibodies against the N-terminus of FAST showed perfect overlap with

antibodies against cytochrome (Li et al., 2004b).

qRT-PCR analysis showed that FASTK1-5 transcripts are expressed in all 13 human

tissues and 11 mouse tissues tested, but different FAST proteins are more abundantly expressed

in mitochondria-enriched tissues. For example, FASTKD1 expression is highest in heart tissue,

and FASTKD3 expression is highest in liver in humans. In mice FASTKD4 is expressed at

highest levels in brown adipose tissue and FASTKD2 levels are highest in testis (Simarro et al.,

2010).

FAST – Fas activated serine/threonine kinase

FAST, the founding member of the FAST protein family is a sensor of mitochondrial

stress that modulates protein translation to promote cell survival. FAST has been shown to

interact with TIA-1. TIA-1 functions to bind the 3'UTR of Fas mRNA to regulate alternative

splicing of the Fas gene. Upon onset of apoptosis via Fas ligation or UV irradiation, FAST is

rapidly dephosphorylated and displaced from the OMM. Displaced FAST binds and phosphorylates cytoplasmic TIA-1 to prevent apoptosis. FAST knockdown promotes exon 6 skipping of endogenous Fas gene. Over-expression of FAST inhibits apoptosis (Li et al., 2004b) (Izquierdo and Valcarcel, 2007).

In addition to two FAST kinase domains and the RAP domain, FAST also contains a BH3-related domain, a putative transmembrane domain and a mitochondrial tethering domain near the c-terminus of the protein (Li et al., 2004a). FAST's BH3-related domain is an amphipathic helix like that found in the pro-apoptotic BH3-only family of proteins. The BH3 domain allows FAST to bind to BCL-XL, a pro-survival protein located at the outer mitochondrial membrane. The FAST mitochondrial tethering domain is rich in lysine and arginine and is structurally similar to the mitochondrial tethering motifs of monoamine oxidase B and cytochrome b5.   FAST truncation mutants lacking a mitochondrial tethering domain (amino acids 368-426) do not bind BCL-XL. It is likely that since FAST does not promote apoptosis it may interact with BCL family members in fundamentally different way (Li et al., 2004a).

FASTKD2

In addition to the requisite components of a FAST family protein, FASTKD2 has one extra putative transmembrane sequence just upstream of the two fast kinase domains. Based on proteinase K digestion analysis and valinomycin studies, it was shown that that FASTKD2 is imported into the inner membrane compartment through the proton-dependent TOM-TIM machinery. It is predicted that FASTKD2 is anchored to the inner mitochondrial membrane and projects into the inter-membrane space (Ghezzi et al., 2008).

Ghezzi and colleagues reported an inherited mitochondrial encephalopathy in two siblings which mapped to a defective FASTKD2 gene. The siblings, a boy and a girl,

experienced normal gestation, uneventful deliveries and unremarkable early development.

However at ages 10 months and 7 months, respectively, following a febrile illness each sibling

developed mitochondrial encephalopathy which was characterized by developmental delay,

progressive hemiplegia, convulsions, asymmetrical brain atrophy and low cytochrome oxidase

(COX) activity in skeletal muscle. Genetic analysis found that the siblings were homozygous for

a nonsense mutation in FASTKD2. The mutation lead to the expression of a truncated

FASTKD2 protein lacking amino acids 278-644, a region including both transmembrane

sequences, the two FAST kinase domains and the RAP domain (Ghezzi et al., 2008).

The same authors found that it was not possible to culture cells over expressing

FASTKD2. In both HeLa cells and COS7 cells this lead to cell body shrinkage, nuclear

fragmentation and apoptotic cell death. Whether this effect is due to a specific function of

FASTKD2, or to a generic toxic effect due to damage of the inner mitochondrial membrane is

not clear, however. Analysis by blue native gel electrophoresis revealed there was no defect in

assembly of any of the mitochondrial respiratory complexes. As COX activity in both patients

was reduced to 14% as normalized to citrate synthase, this suggests that FASTKD2 may be

indirectly involved with the function of COX (Ghezzi et al., 2008).

A 2011 study of apoptotic pathways in breast cancer cells showed that FASTKD2

expression is regulated by the NRIF3/DD1 pathway. ChIP studies indicated that the DIF-1

complex binds the 5' untranslated first exon of the FASTKD2 mRNA. Either phosphorylation of

Ser28 of differentiation-inducing factor-1 (DIF-1) or an increase in the co-regulator of nuclear

hormone receptor NRIF3 levels lead to the release of repression of FASTKD2 and then to

apoptosis. Interestingly, FASTKD2 expression leads to apoptosis but does not require

mitochondrial localization.   A vector expressing FASTKD2 lacking the N-terminal

mitochondrial import signal and found that FASTKD2 can mediate apoptosis independent of mitochondrial importation. Thus, it may be that a rapid and transient increase in FASTKD2 expression can cause apoptosis possibly via phosphorylation, leading to activation of proapoptotic factors or inhibition of antiapoptotic factors that reside on the surface or outside of the mitochondria (Yeung et al., 2011).

FASTKD3

Tandem Affinity Purification (TAP) revealed that FASTKD3 interacts with many components of mitochondrial respiratory and translation machinery. 66 proteins were found to co-purify with FASTKD3 (Simarro et al., 2010). Such a large number of interacting proteins suggests that FASTKD3 may function in the mitochondria as part of a complex. Among the proteins tandem-affinity purified with FASTKD3 were 54 known mitochondrial proteins, including nucleoid proteins involved in mtDNA replication and transcription, proteins involved in oxidative phosphorylation, lipid metabolism, the citric acid cycle, amino acid catabolism, RNA processing, translation, some non-mitochondrial proteins and proteins with unknown function, FASTKD2 - a fellow FAST family member, and LRPPRC, a mitochondrial RNA binding protein (Simarro et al., 2010). siRNA knockdown of FASTKD3 and analysis by western blot with antibodies probing for subunits of each mitochondrial respiratory complexes revealed no defect in the formation of any of the complexes. Additionally, siRNA knockdown has no effect on the levels of cellular mtDNA, cell viability, proliferation (Simarro et al., 2010).

However, siRNA knockdown of FASTKD3 resulted in a dramatic 57% decrease in overall cellular respiration as measured by oxygen consumption U2OS cells. The addition of the ATP synthase inhibitor oligomycin allowed quantification of ATP-coupled respiration. Knockdown cells showed 39.4% of the capacity of control cells. To assess the maximal

respiratory capacity, the MRC uncoupler FCCP was added to the cells.   Both the direct

measurement of OCR and the percent increase over baseline in response to FCCP were

significantly lower, as compared with control-siRNA treated cells. The addition of the complex I

inhibitor rotenone resulted in a further reduction in OCR values to 15% of baseline in control-

siRNA treated cells and 20% of baseline in FASTKD3-siRNA treated cells.   These results

suggest an impairment of the reserve respiratory capacity in FASTKD3-siRNA treated cells

which would result in mitochondrial dysfunction in tissues with high metabolic demand. The

adverse effects on cellular respiration in FASTKD3 knockdown cells taken together with the

capacity of knockdown cells to form functional MRC complexes it is possible that the reduction

in respiration may be due to a specific function of FASTKD3 (Simarro et al., 2010).

FASTKD4

The FASTKD4 protein has been the focus of my research in the laboratory of Dr. Dan

Bogenhagen since September 2011.   Our lab became interested in FASTKD4 in 2006 when a it

was identified as a suspected contaminant of purified mtDNA nucleoids separated on a

sedimentation gradient (Wang and Bogenhagen, 2006). FASTKD4 has been referenced in only

one scientific publication, in which the authors made a brief structural analysis based on its

amino acid sequence and compared its phylogenetic position within the FASTK family.

However, in this report there was no experimental analysis performed in order to characterize the

function of FASTKD4 (Li et al., 2004b). Following is a summary of what we have learned in

our work to characterize FASTKD4.

Materials and Methods

Protein sequence alignment

Protein sequence alignments were performed using Vector NTI software version 11.5 (Invitrogen) using FASTKD4 NCBI Reference Sequence: NP_004740.2 and FAST NCBI Reference Sequence: NP_006703.

Transmembrane sequence prediction

Putative transmembrane sequences were identified in FASTKD4 utilizing the FASTA amino acid sequence for FASTKD4 NCBI Reference Sequence: NP_004740.2 with the TMpred - Prediction of Transmembrane Regions and Protein Orientation (EMBnet-CH) software located at http://www.ch.embnet.org/software/TMPRED_form.html   (Hoffman & Stoffel, 1993).

Cell culture

3T3-Switch mouse fibroblasts were obtained from Invitrogen and cultivated in Dulbecco's Modified Eagle Medium (Gibco) containing 10% Fetal Calf Serum, 1% penicillin and streptomycin, and hygromycin 50 µg/ml. To remove cells from tissue culture plates, the cell layer was briefly rinsed with 2 ml 1X PBS. 1.0 ml 0.25% (w/v) Trypsin-0.5 mM-EDTA was added and cells were incubated at 37°C for two minutes. 4.0 to 6.0 ml of medium was added and cells were resuspended by pipetting. Aliquots of suspended cells were added to new culture plates. Cultures were incubated at 37°C with 5% CO_2.

Proteinase K digestion

Proteinase K digestion was performed according to protocol as in (Bogenhagen et al., 2008).

cDNA cloning

cDNA was obtained from mouse tissue first-strand cDNA libraries (Clontech). Forward and reverse primers were designed to generate a FASTKD4 DNA fragment (1913 nt) flanked by Asc1 and Not1 restriction endonuclease sites. The forward primer sequence:

5'-C**GGCGCGCC**AGAA<u>ATG</u>GCCGTTCGCCTGATG-3' The first codon "ATG" (underlined) corresponds to the first amino acid of the coding sequence and is preceded by the Asc1 restriction endonuclease site (noted in bold). The reverse primer sequence 5'-**TGCGGCCGC**ATTTGGCTAGCTCC-3' contains the NotI enzyme restriction endonuclease site. The PCR reaction was performed in a total volume of 40 µl including 20 µl 2X Phusion HF Buffer (New England Biolabs), 20 pm FASTKD4 forward primer, 20 pm FASTKD4 reverse primer, 1 µl mouse cDNA, 17 µl dH2O. Amplification was conducted on a Mastercycler Gradient (Eppendorf) using the following conditions: initial denaturation for 2 min at 98°C, denaturation 15 sec at 98°C, annealing 20 sec at 68.1°C, elongation for 45 sec at 72°C. The cycle was repeated 29 times with a final elongation step of 5 min at 72°C. To confirm successful amplification the PCR products were resolved by gel electrophoresis in 1.0% agarose gel (Invitrogen) and stained with ethidium bromide (MP Biomedicals). The band corresponding to the desired FASTKD4 fragment (1913 nt) was excised from the gel and purified using the MinElute Gel Extraction Kit (Qiagen).

The cDNA fragment was ligated into the pCRII vector using the TOPO Cloning system (Invitrogen) for further amplification. This reaction was performed as per manufacturer's protocol. One Shot Chemically Competent *E. coli* TOP10 cells (Invitrogen) were transformed with this FASTKD4-TOPO ligation. Transformed cells were then spread on 1.5% agar plates with kanamycin (50 mg/l) (Sigma-Aldrich) and incubated overnight at 37°C.

The following day, colonies were selected and expanded overnight in 3.0 ml 2XYT (Fisher Scientific) liquid medium with kanamycin 50 mg/l at 37°C in an orbital shaker incubator. To affirm that the ligation proceeded correctly, DNA from 1.0 ml of each culture was extracted using a QIAprep Spin Miniprep Kit (Qiagen). 500 µg DNA from each culture was digested using 1.0 µl of each restriction endonuclease in a total reaction volume of 20 µl for 1 hour. The DNA was resolved by 1% agarose gel electrophoresis for confirmation of positive transformation. To verify that the sequence of the TOPO clone was correct, a 300 ng sample of each FASTKD4 pCR product with forward and reverse primers were delivered to Genomic Core Facility at Stony Brook University for sequencing. The results of the sequencing were compared to Genbank sequences for mouse FASTKD4 for accuracy. A mutation (GA**G** to GA**A)** was detected at position 1396. Since this is a silent mutation in that both codons in specify glutamic acid, the sequence was determined to be good.

Plasmid - pGS-FASTKD4-EOS

To insert the FASTKD4 DNA fragment into the pGS-EOS Expression vector a 500 µg sample of purified extracted FASTKD4-TOPO DNA was digested using 1.0 µl each of restriction endonucleases Asc1 and Not1 in a total volume of 20 µl. The restriction digest was resolved by gel electrophoresis in 1.0% agarose gel and stained with ethidium bromide to confirm correct digestion. Bands corresponding to the FASTKD4 fragment (1913 nt) were excised from the gel and purified using the MinElute Gel Extraction kit (Qiagen). To insert the FASTKD4 DNA fragment into the pGS-EOS vector, a ligation reaction was prepared: 300 µg purified FASTKD4 DNA was combined with 150µg pGS-EOS vector, 1µl 10x buffer and 1µl T4 DNA Ligase for a total reaction volume of 10 µl and incubated at 4°C overnight.

3 µl of pGS-FASTKD4-EOS ligation product was added to one half aliquot of thawed

One Shot Top10 chemically competent E. coli cells and incubated on ice for 40 minutes. The

cells were then heat shocked at 42°C for 35 seconds. Immediately after heat shocking, 300 µl of

S.O.C. medium warmed to 37° was added and incubated for 1 hour and 45 minutes in a 37°C

shaker. 200 µl of transformed cells were plated onto 1.5% agar plates with ampicillin 50 mg/l,

and incubated overnight at 37°C.

Colonies from each agar plate were selected and expanded overnight in 3.0 ml 2XYT

liquid medium containing ampicillin 50 µg/ml at 37°C in an orbital shaker. DNA from 1.0 ml of

each culture was extracted using a QIAprep Spin Miniprep Kit (Qiagen). 500 ng purified DNA

from each sample was digested using 1.0 µl each of restriction endonucleases Asc1 and Not1 in a

total volume of 20 µl. The restriction digest was resolved by gel electrophoresis in 1.0% agarose

gel and stained with ethidium bromide. Appropriately sized band was cut out and eluted from

agarose gel with MinElute kit (Qiagen) to isolate DNA fragments.

Plasmid - pGS-FASTKD4-3FH

Two restriction digests were prepared. 500 ng of pGS-TFAM-3FH DNA was digested

using 1.0 µl each of restriction endonucleases Asc1 and Not1 in a total volume of 20 µl. 500 ng

of pGS-FASTKD4-EOS DNA was digested using 1.0 µl each of restriction endonucleases Asc1

and Not1 in a total volume of 20 µl. Both digestion products were resolved by gel

electrophoresis in 1.0% agarose gel and stained with ethidium bromide. Bands corresponding to

the FASTKD4 DNA fragment (1900 nt) and the pGS-3FH vector (5731 nt) were cut out and

eluted from agarose gel with MinElute kit. To insert FASTKD4 DNA into the 3FH vector a

ligation reaction was prepared: 2 µl purified FASTKD4 DNA was combined with 3 µl purified

pGS-EOS vector, 1μl 10x buffer and 1μl T4 DNA Ligase for a total reaction volume of 10 μl

and incubated at 4°C overnight.

To transform TOP 10 electro chemically competent E. coli cells with the FASTKD4-3FH

vector, 2 μl of the ligation product was transferred to a cuvette and subjected to electroporation.

The product was transferred to a 40 ml conical tube, combined with 1 ml of 2XYT liquid

medium and incubated at 37°C for 1 hour. 50 and 100 μl aliquots of transformed cells were

plated onto 1.5% agar plates with ampicillin 50 mg/l, and incubated overnight at 37°C. Two

colonies were selected and expanded in 4ml 2XYT liquid medium with ampicillin 50 mg/l at

37°C overnight in an orbital shaker.   DNA from 1.0 ml of each culture was extracted using a

QIAprep Spin Miniprep kit (Qiagen). 500 μg DNA from each culture was digested using 1.0 μl

each of restriction endonucleases Nde1 and Not1 in a total volume of 20 μl. Digestion products

were resolved by gel electrophoresis in 1.0% agarose gel and stained with ethidium bromide.

To amplify the FASTKD4-3FH DNA, 100 ml of 2XYT liquid medium with ampicillin 50

mg/l were inoculated with 100 μl of transformed TOP 10 cells and incubated overnight at 37°C

in an orbital shaker. DNA from the 100 ml culture was extracted using a Plasmid Plus Midi Kit

(Qiagen).

Plasmid pGS-FASTKD4-EOS

Forward and reverse primers were designed to generate a FASTKD4 DNA fragment

(1900 nt) flanked by Nde1 (forward) and Not1 (reverse) restriction endonuclease sites.

The forward primer sequence: 5'-**GCAT<u>ATG</u>G**CTGGAGTGTCCTGC-3.' The first codon

"ATG" (underlined) corresponds to the first amino acid of the coding sequence and is included

in the Nde1 enzyme restriction endonuclease site (noted in bold).

The reverse primer sequence: 5'-T**GCGGCCGC**ATTTGGCTAGCTCC-3' The Not1 enzyme

restriction endonuclease site is noted in bold.

The PCR reaction was performed in a total volume of 40 µl including 10 µl 20X Phusion HF Buffer (New England Biolabs), 20 pm FASTKD4 forward primer, 20 pm FASTKD4 reverse primer, 1 µl Mouse cDNA library, 17 µl dH2O. Amplification was conducted in a Mastercycler Gradient (Eppendorf) using the following conditions: Initial denaturation was for 2 min at 98°C, denaturation 15 sec at 98°C, annealing 20 sec at 68.1°C, elongation for 45 sec at 72°C. The cycle was repeated 29 times with a final elongation step of 5 min at 72°C.   To confirm successful amplification the PCR products were resolved by gel electrophoresis in 1.0% agarose gel and stained with ethidium bromide. The band corresponding to the desired FASTKD4 fragment (1913 nt) were excised from the gel and purified using the MinElute Gel Extraction kit (Qiagen).

Plasmid - pET-22b+ FASTKD4-6XHis

To insert the gene for FASTKD4 into the pET-22b+ vector forward and reverse primers were designed to generate a FASTKD4 DNA fragment flanked by NdeI and Not1 restriction endonuclease sites. The forward primer sequence:

 5'- **GCATATG**GCTGGAGTGTCCTGC- 3' The sequence CATATG corresponds to Nde1 restriction endonuclease site (noted in bold) and is designed to anneal to the template in a position that eliminates the mitochondrial localization signal from the FASTKD4 gene. The reverse primer sequence:

5'- T**GCGGCCGC**TTGGCTAGCTC - 3' contains the NotI enzyme restriction endonuclease site GCGGCCGC and is noted in bold. The PCR reaction was performed in a total volume of 40 µl including 20 µl 2X Phusion HF Buffer (New England Biolabs), 20 pm FASTKD4 forward primer, 20 pm FASTKD4 reverse primer, 500 ng pGS-FASTKD4-EOS DNA, and 17 µl dH2O.

Amplification was conducted on a Mastercycler Gradient (Eppendorf) using the following conditions: initial denaturation for 2 min at 98°C, denaturation 15 sec at 98°C, annealing 20 sec at 68.1°C, elongation for 45 sec at 72°C. The cycle was repeated 29 times with a final elongation step of 5 min at 72°C.

To confirm successful amplification the PCR products were resolved by gel electrophoresis in 1.0% agarose gel (Invitrogen) and stained with ethidium bromide (MP Biomedicals). The band corresponding to the desired FASTKD4 fragment (1813 nt) was excised from the gel and purified using the MinElute Gel Extraction Kit (Qiagen).

To insert the FASTKD4 DNA fragment into the pET-22b+ vector a ligation reaction was prepared. 200 ng of FASTKD4 DNA and 400 ng of Pet22-6XHis vector DNA were combined with 1.0 μl T4 DNA ligase to a total volume of 10 μl and incubated at 4°C overnight. 2.0 μl of the ligation reaction were added to one aliquot of One Shot Top10 chemically competent E. coli cells and incubated on ice for 45 minutes. The cells were then heat shocked at 42°C for 30 seconds. Immediately after heat shocking, 300 μl of S.O.C. liquid medium warmed to 37° was added and the cells incubated for 1 hour in a 37°C orbital shaker. 200 μl of transformed cells were plated onto 1.5% agar plates with kanamycin 50 mg/l (Sigma-Aldrich) and incubated overnight at 37°C. The following day, colonies were selected and expanded overnight in 3.0 ml 2XYT liquid medium with kanamycin 50 mg/l at 37°C in an orbital shaker incubator. DNA from 1.0 ml of each culture was extracted using a QIAprep Spin Miniprep kit (Qiagen). 500 μg DNA from each culture was digested using 1.0 μl of each restriction endonuclease in a total volume of 20 μl. Digestion products were resolved by gel electrophoresis in 1.0% agarose gel and stained with ethidium bromide. A 100 μl sample of FASTKD4-Pet22 culture was selected

for overnight expansion in 100 ml 2XYT liquid medium with ampicillin 50 mg/ml. DNA from

100 ml of the culture was extracted using a Plasmid Plus Midi Kit (Qiagen).

Transfection of BL21 RIPL cells with FASTKD4-Pet22-6XHis

1 µl of FASTKD4-Pet22-6XHis DNA was added to 33 µl BL21 RIPL electro chemically

competent *E. coli* cells. The mixture was then subjected to electroporation, plated onto 1.5%

agar plates with chloramphenicol 50 mg/l and ampicillin 50 mg/l, inverted and incubated

overnight at 37°C.   Two colonies were selected and expanded in 4 ml 2XYT liquid medium

with chloramphenicol 50 mg/l and ampicillin 50 mg/l at 37°C overnight in an orbital shaker.

DNA from 1.0 ml of each culture was extracted using a QIAprep Spin Miniprep Kit (Qiagen).

500 µg DNA from each culture was digested using 1.0 µl each of restriction endonucleases Nde1

and Not1 in a total volume of 20 µl. Digestion products were resolved by gel electrophoresis in

1.0% agarose gel and stained with ethidium bromide.

Transfection of mammalian cells

1.0 ml aliquot of 3T3-Switch (Invitrogen) cells were thawed and plated on 6 cm tissue

culture plate in 10 ml DMEM liquid medium, incubated at 37°C and monitored for viability over

a period of 3 days. Either pGS-FASTKD4-EOS DNA or pGS-FASTKD4-3FH DNA was further

purified by phenol-chloroform extraction and ethanol precipitation.   3 µg purified DNA were

treated with FuGene HD transfection reagent (Roche) according to the manufacturer's protocol.

The treated DNA was then applied to 3T3-Switch cells previously seeded on a 6 cm tissue

culture plate in DMEM liquid medium and incubated overnight at 37°C. As a control one 10cm

plate of 3T3-Switch cells was left untransformed. The Zeocin (Invitrogen) concentration in the

DMEM liquid medium was gradually increased to 140 µg/ml to select for stably-transformed

cells.

Clonal selection of transformed mammalian cells

Six clonal colonies of transformed cells growing at 140 µg/ml Zeocin were selected for expansion. One 0.75 cm sterile filter paper disk was laid over each colony and 25 µl 0.05% trypsin-EDTA (Gibco) was applied. The trypsinized colonies were incubated at 37°C for 2 minutes. The six filter paper disks were grasped with sterile forceps, pushed back and forth three times, removed to an individual well in a six well tissue culture plates containing 1.0 ml each of complete DMEM liquid medium, incubated at 37°C, monitoring for cell viability.   The concentration of Zeocin was gradually increased to 160 µg/ml. Two colonies survived and were named Clones 1 and 2.   Both clone cultures and the original mass culture cells were maintained in 200 µg/ml Zeocin.

IPTG induction of BL21 RIPL cells

200 µl BL21 RIPL FASTKD4-Pet22 transformed cells were placed into 200 ml 2XYT liquid medium with chloramphenicol 50 mg/l and ampicillin 50 mg/l. 200 µl of 1M IPTG was added to one of the cultures to a final concentration of 1 mM. Both the IPTG induced and non-induced cultures were incubated at 16°C overnight. The following day two 200 µl aliquots of BL21 RIPL FASTKD4-Pet22 transformed cells were placed into 200 ml 2XYT liquid medium with chloramphenicol 50 mg/l and ampicillin 50 mg/l. The cultures were monitored to an optical density at 600 nanometers of 0.35. 200 µl of 1M IPTG was added to one of the cultures to a final concentration of 1 mM. Both induced and non-induced cultures were incubated at 37°C for 3 hours. Following the induction period cell cultures was pelleted 5 min at 5,000 rpm and resuspended in P150 buffer with TX-100 and sonicated to obtain a total cellular lysate fraction. The sample was re pelleted and the supernatant was drawn off to obtain a soluble lysate fraction.

Mifepristone induction of transfected mammalian cells

To induce expression of FASTKD4 fusion protein, mifepristone was applied to tissue culture plates of cells grown to approximately 65% confluence in DMEM to a final concentration of 50 pM. The cells were incubated overnight at 37°C.

Confocal imaging of induced transfected mammalian cells

To visualize the cell cultures using immunofluorescent microscopy, cells were seeded in four chambered cover slips treated with fibronectin and incubated overnight in DMEM. The next evening, mifepristone was added to a final concentration of 50 pM and cells were incubated 14 hours at 37°C. Mitotracker Red (Life Technologies) was added to a final concentration of 10 uM and incubated at 37°C for one hour. The cells were fixed with 4% paraformaldehyde and incubated at room temperature for 30 minutes. The cells were washed three times briefly with 1X PBS with 0.25% TX-100. 1 ml MaxBlock (Active Motif) was added and the cells were incubated at 4°C overnight or 20 minutes at room temperature on an orbital shaker. The cells were blocked with a solution of 1X PBS containing 0.2% TX-100, 5% horse serum, 3% BSA. The blocking solution was aspirated and rabbit anti-Flag primary antibody was applied to a final concentration of 0.05%. Cells were incubated in darkness for one hour up to overnight on an orbital shaker at room temperature. Cells were washed three times for ten minutes with 1X PBS and 0.2% TX-100. The cells were incubated in darkness at room temperature on an orbital shaker for one hour in blocking solution containing the fluorescent secondary antibody goat anti-rabbit Alexa Fluor 488 (Invitrogen) to a final concentration of 0.1%. The secondary antibody was aspirated and the cells were briefly washed three times with 1X PBS. Cells were imaged using a Zeiss LSM 510 Confocal microscope (Carl Zeiss, Inc.).

Purification of mitochondria from cell culture

Twelve 15 cm plates of 3T3 cells stably transfected with pGS-FASTKD4-3FH were induced and collected from tissue culture plates according to the induction and cell culture protocol above. Cells were counted using hemacytometer (Fisher Scientific) and pelleted in a 50 ml conical tube by centrifugation at 500 xg for 5 min. The pellet was rinsed in 1X PBS and re-pelleted by centrifugation at 500 xg. The pellet was resuspended in 4 ml hypotonic buffer containing 20 mM Hepes pH 7.4 and 0.1% by volume of each of the protease inhibitors 0.2 M PMSF, 5 mg/ml leupeptin and 1 mM pepstatin. The cells were incubated on ice and monitored for swelling. 50 μl 8% CHAPS was added and the cells were vortexed at maximum speed for 5 sec. The cells were then passed through a ⅝ inch 25 gauge needle several times and 3.2 ml 2X MSH+EDTA was added and the tube was inverted several times to mix to yield a cell homogenate. Microscopic inspection was used to confirm cell lysis. The cell homogenate was centrifuged at 1,200 xg for 5 min at 4°C to remove nuclear debris to yield post nuclear supernatant. Post nuclear supernatant was centrifuged at 10,000 xg for 15 min at 4° C to yield a crude mitochondrial pellet. The crude mitochondrial pellet was resuspended in 1.0 ml 1X MSH+EDTA, layered over a 1.7M / 1.0M sucrose gradient and centrifuged in a SW41 swinging bucket rotor at 25,000 rpm for 25 minutes at 4°C. The purified mitochondria were collected from the sucrose gradient interface, diluted in 3 volumes of 0.5X MSH, centrifuged at 10,000 xg for 15 minutes at 4°C to obtain a purified mitochondrial pellet. The pellet was resuspended in 1.0 ml 1X MSH+EDTA, re-pelleted at 10,000 xg, the supernatant removed and the pellet was stored at -80°C.

Tandem affinity purification of FASTKD4-3FH from purified mitochondria

Purified mitochondria containing the fusion protein FASTKD4-3FH were washed in 1X Buffer containing 5 mM Tris pH 7.4, 15 mM NaCl and 0.1% by volume of each of the protease inhibitors 0.2 M PMSF, 5 mg/ml leupeptin and 1 mM pepstatin. Mitochondrial 2X lysis buffer was added to a final concentration of 50 mM Hepes pH 7.5, 100 mM NaCl, 2% TX-100 and .1% each of the protease inhibitors PMSF, leupeptin and pepstatin. 50 µl mouse anti-FlagM2 agarose beads (Sigma) were prepared according to manufacturer's protocol. Lysed mitochondria were applied to the prepared beads and incubated 3 hours on a rotator at 4°C. The beads were then washed 5X with 1X wash buffer containing 0.02% TX-100 and 0.1% by volume of each of the protease inhibitors 0.2 M PMSF, 5 mg/ml leupeptin and 1 mM pepstatin, and eluted 5X with elution buffer containing 150 ng/µl 3X FLAG solution and 0.02% TX-100 and 0.1% by volume of each of the protease inhibitors 0.2 M PMSF, 5 mg/ml leupeptin and 1 mM pepstatin. All fractions were stored at -80°C.

Elution fractions 1 – 3 from the FlagM2 immunoprecipitation were applied to 100 µl His60 Ni Superflow resin beads (Clontech) which had been prepared according to the manufacturers protocol and incubated for 2 hours on a rotator at 4°C. The beads were then washed 3X with buffer containing 0.02% TX-100, 2.0 mM 2-mercaptoethanol and 0.1% by volume of each of the protease inhibitors 0.2 M PMSF, 5 mg/ml leupeptin and 1 mM pepstatin. The beads were then eluted 3X with elution buffer containing 0.02% TX-100, 2.0 mM 2-mercaptoethanol, 1M imidazole and 0.1% by volume of each of the protease inhibitors 0.2 M PMSF, 5 mg/ml leupeptin and 1 mM pepstatin.

Affinity purification of FASTKD4-6XHis with nickel column

The soluble lysate from the FASTKD4-6XHis transfected cells was used for affinity purification of the tagged protein using a GE Healthcare 5 ml HisTrap nickel resin column. Buffers were prepared: Load and wash Buffer A contained 20 mM Tris pH 8.4, 100 mM KCl, 1 mM EDTA, 5 mM BME, 5.0% glycerol, 0.02% TX-100, 0.1% by volume 0.2 M PMSF, 5 mg/ml leupeptin, 1 mM pepstatin and de-ionized water to a final volume of 200 ml and filtered. Elution buffer B contained 20 mM Tris pH 8.4, 1 mM EDTA, 7 mm EDTA, 5.0% glycerol, 0.02% TX-100, 0.1% by volume 0.2 M PMSF, 5 mg/ml leupeptin, 1 mM pepstatin and de-ionized water to a final volume of 200 ml and filtered. Soluble cellular lysate in Buffer A was loaded into the column. The affinity-bound protein was eluted with 200 mM imidazole. Fractions were analyzed by SDS Page and Coomassie (Bio-Rad) staining.

Results and Discussion

Protein sequence alignment - FAST BH3 domain vs. FASTKD4

In addition to the requisite features of a FAST Family kinase which are two FAST kinase

domains and a RAP domain, FASTKD4 has a BH3-like domain similar to the BH3-like domain

in FAST, the founding member of the FAST family of proteins. Figure 1 shows an alignment

using Vector NTI software (Invitrogen) of the amino acids which comprise the BH3-like domain

of FAST and the analogous region in FASTKD4 (residues 498-512). Sequence conservation is

evident as green highlights indicate functionally conserved residues and yellow highlights

indicate sequence identity. This might indicate that FASTKD4 plays a role in apoptosis.

Identification of FASTKD4 protein binding partners may help elucidate this role.

Figure 1 - Alignment of FAST BH3 domain with analogous region of FASTKD4

Putative transmembrane sequence prediction

The amino acid sequence of FASTKD4 was analyzed using the TMPred - Topology

prediction of membrane proteins server located at:

http://www.ch.embnet.org/software/TMPRED_form.html

Using the NCBI FASTA amino acid sequence for human FASTKD4 isoform 1 (NCBI

Reference Sequence: NP_004740.2) , the server predicted two potential transmembrane domains

in FASTKD4 as indicated outlined in green squares below (Figure 2) spanning amino acids 127-

146, and 582-605 (Hofmann, 1993).   The presence of two putative transmembrane segments

may indicate that FASTKD4 is anchored in one of the mitochondrial membranes, with both the

N and C termini projecting either into the matrix or the inter-membrane space. Further analysis

with methods such as sodium carbonate extraction of proteins from sonicated purified

mitochondrial membranes may help to confirm FASTKD4's residence within mitochondrial
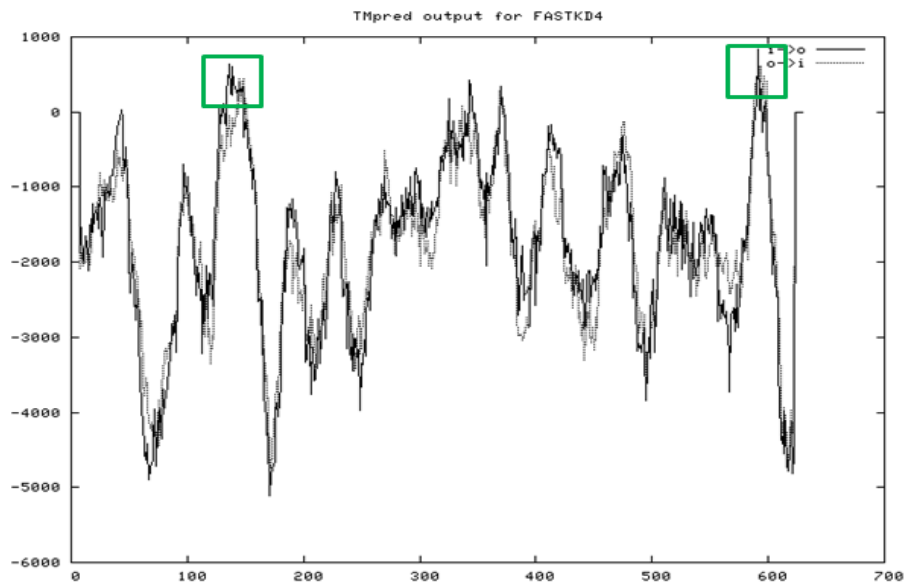
membrane.

Figure 2 - TMPred prediction of transmembrane regions - FASTKD4

Western blot analysis of HeLa cell fractions

To determine the cellular localization of FASTK4, SDS-page and Western blot analysis were performed on previously prepared samples of HeLa cell fractions. Cell homogenate, post nuclear supernatant, crude mitochondria and purified mitochondria fractions were probed for FASTKD4 (Figure 3). The results show that FASTKD4 is present in all fractions and is enriched in mitochondria.
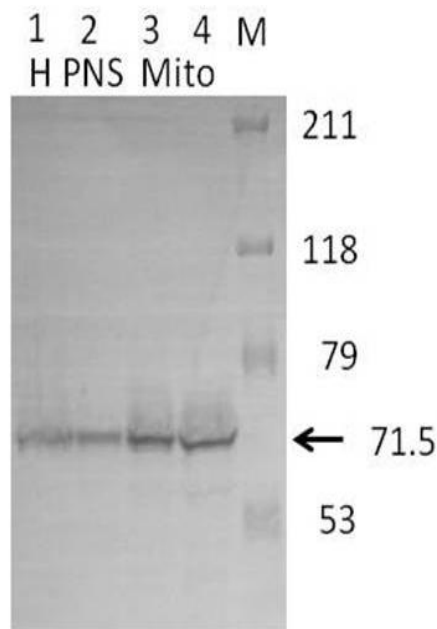

Figure 3 - Western blot analysis of HeLa cell fractions

Figure 3: 40 µg of protein each from previously prepared HeLa cell fractions were analyzed by 10% SDS-page followed by Western blotting. Primary antibody: rabbit anti-FASTKD4. Secondary antibody: goat anti-rabbit phosphatase labeled. Lane 1 contains the homogenate; lane 2 contains the post-nuclear supernatant and lanes 3 and 4 contain crude and highly purified mitochondria, respectively.

Proteinase K digestion

        To determine whether FASTKD4 is an outer mitochondrial membrane protein or resides

within the mitochondria, a time sequence proteinase K digestion was performed. Previously

prepared purified HeLa cell mitochondrial fractions were analyzed by western blotting with

primary antibodies for FASTKD4 and Tom20, a known outer mitochondrial membrane protein.

As the image below illustrates, FASTKD4 is present in mitochondria after 20 minutes exposure

to Proteinase K, while Tom 20 was not. This is an indication that FASTKD4 resides within the

mitochondria and has been protected by the mitochondrial membranes (Bogenhagen, 2011 -

unpublished results). Furthermore, the bands on the Western blot indicating FASTKD4 do not

change position. This suggests that there has been no reduction in size from digestion by

proteinase K. A band shift resulting from a change in size might be expected if FASTKD4

inserts in the OMM in such a way that a portion or portions of it are exposed on the outer surface

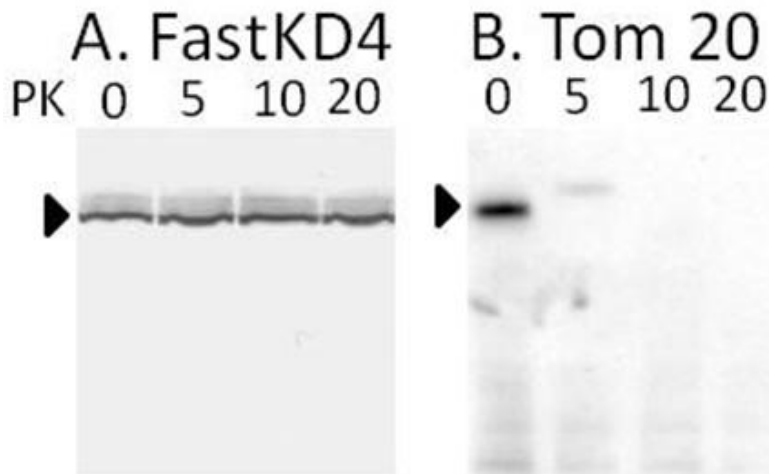of the mitochondria are thus subjected to partial digestion by proteinase K.

Figure 4 - FASTKD4 Proteinase K digestion (HeLa cells)

        Figure 4: Purified mitochondria from HeLa cells were subjected to a twenty minute
digestion with proteinase K. Fractions at five, ten and twenty minutes were analyzed by SDS-
Page and western blot. Primary antibodies: rabbit anti-FASTKD4 and rabbit anti-Tom20.
Secondary antibody: goat anti-rabbit phosphatase labeled.

IPTG induced expression and solubility test in BL21 RIPL *E. coli*

   To produce large quantities of pure FASTKD4 protein, the FASTKD4 gene was ligated

into the pET-22b+ vector (Figure 5) which confers a c-terminal 6XHis tag, an IPTG inducible

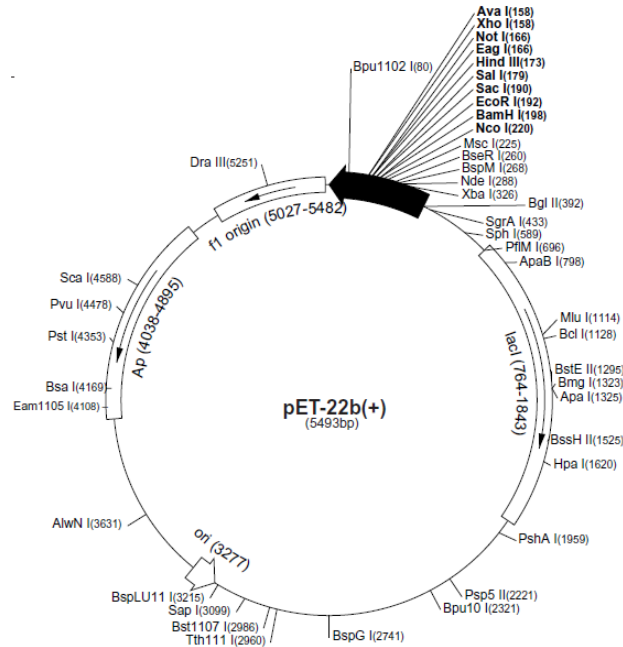promoter and a gene for resistance to ampicillin.

Figure 5 - pET-22b(+) vector

   The ligation product was then transfected into BL21 RIPL *E. coli* cells, cultured and

subjected to selection by chloramphenicol and ampicillin. Positive transformants were

confirmed by NdeI and NotI digest, and expanded in liquid medium. Two IPTG inductions were

performed, one at 16°C overnight and one at 37°C for three hours. These inductions were

accompanied by non-induced controls at identical conditions to measure background expression.

Cells from each experiment were collected by centrifugation, lysed and the total soluble cellular

protein was extracted and analyzed by SDS-PAGE with Coomassie blue staining (Figure 6). It is

evident from the stained gel that IPTG expression of the FASTKD4-6XHis fusion protein is well

controlled, it expresses equally well at both temperatures and is soluble. As there was no

difference in the level of FASTKD4-6XHis fusion protein in cultures induced at 16°C and 37°C,

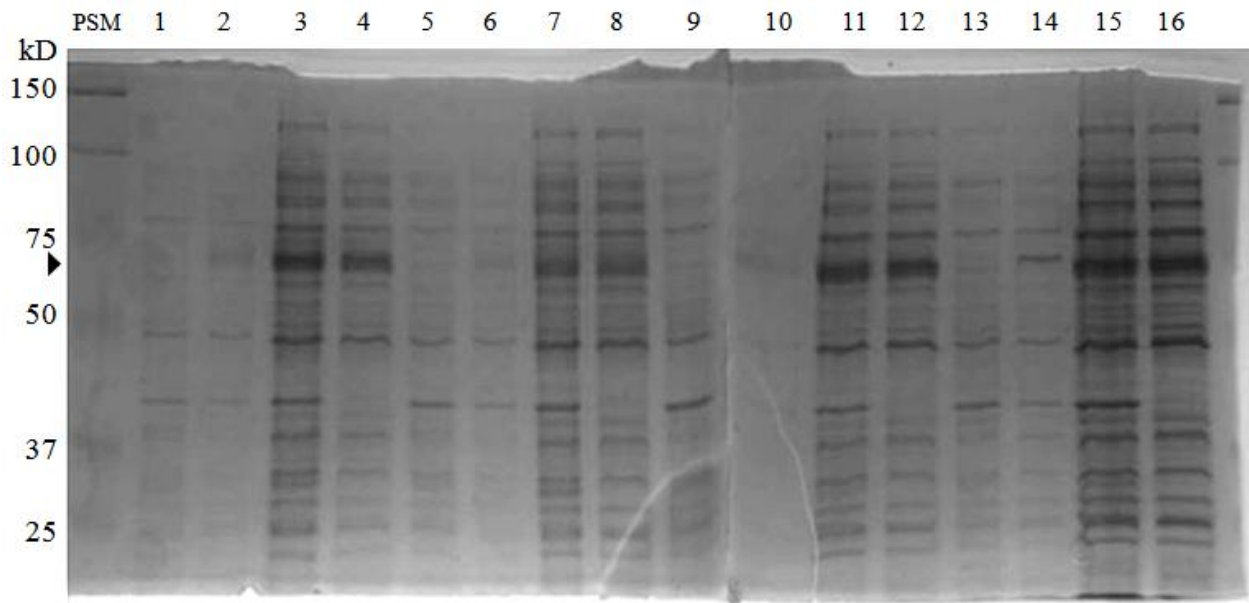the 16°C culture was selected for use in subsequent experiments.

Figure 6 - Induction and solubility test - FASTKD4-6XHis fusion protein

Figure 6: Bacteria-expressed total soluble cellular lysate in induced and non-induced cells at 16°C and 37°C, stained with Coomassie blue. Lanes 1-8 contain fractions of cells grown at 16°C. Lanes 9-16 contain fractions of cells grown at 37°C. Lanes 1, 5, 9, and 13 contain total cell lysate. Lanes 2, 6, 10 and 14 contain total cellular lysate from induced cells. Lanes 3, 7, 11 and 15 contain total cell lysate from induced cells at 8X concentration. Lanes 4, 8, 12 and 16 contain total soluble cellular lysate at 8X concentration. PSM designates the pre-stained marker protein ladder here and in other figures.

Western blot analysis - total soluble cell lysate from non-induced and induced cells

Western blot analysis was performed with total soluble cell lysate fractions from non-induced cells at 37°C and induced cells at 16°C with antibodies probing for FASTKD4. The results show an absence of FASTKD4 in non-induced cells and its presence in the induced cells indicating that the plasmid does not have significant leaky expression (Figure 7.)
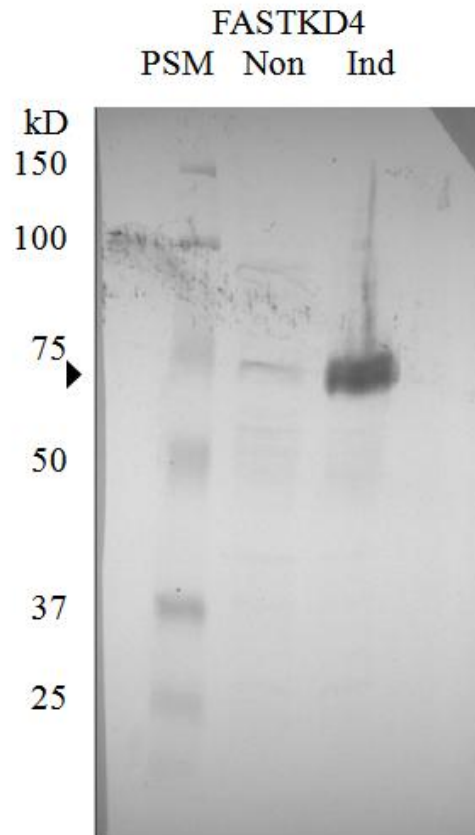

Figure 7 - Western blot analysis of 16°C IPTG induction

Figure 7: Bacteria-expressed total soluble cell lysate in cells induced and non-induced cells grown at 16°C. SDS-page on 10% polyacrylamide gel followed by Western blot analysis. Primary antibody: rabbit anti-FASTKD4. Secondary antibody: goat anti-rabbit phosphatase labeled.

Affinity purification of FASTKD4-6XHis

FASTKD4-6XHis fusion protein was purified from the total soluble cellular protein extracted from cells induced at 16°C overnight on a nickel affinity column and eluted with buffer containing 200 mM imidazole. Elution fractions were analyzed by SDS-PAGE and Coomassie blue staining (Figure 8). The gel image below shows that FASTKD4 eluted well from the nickel resin affinity column as the imidazole concentration reached 200 mM, and was no longer present in significant quantities after fraction 21 as the elution buffer concentration reached 100% imidazole.
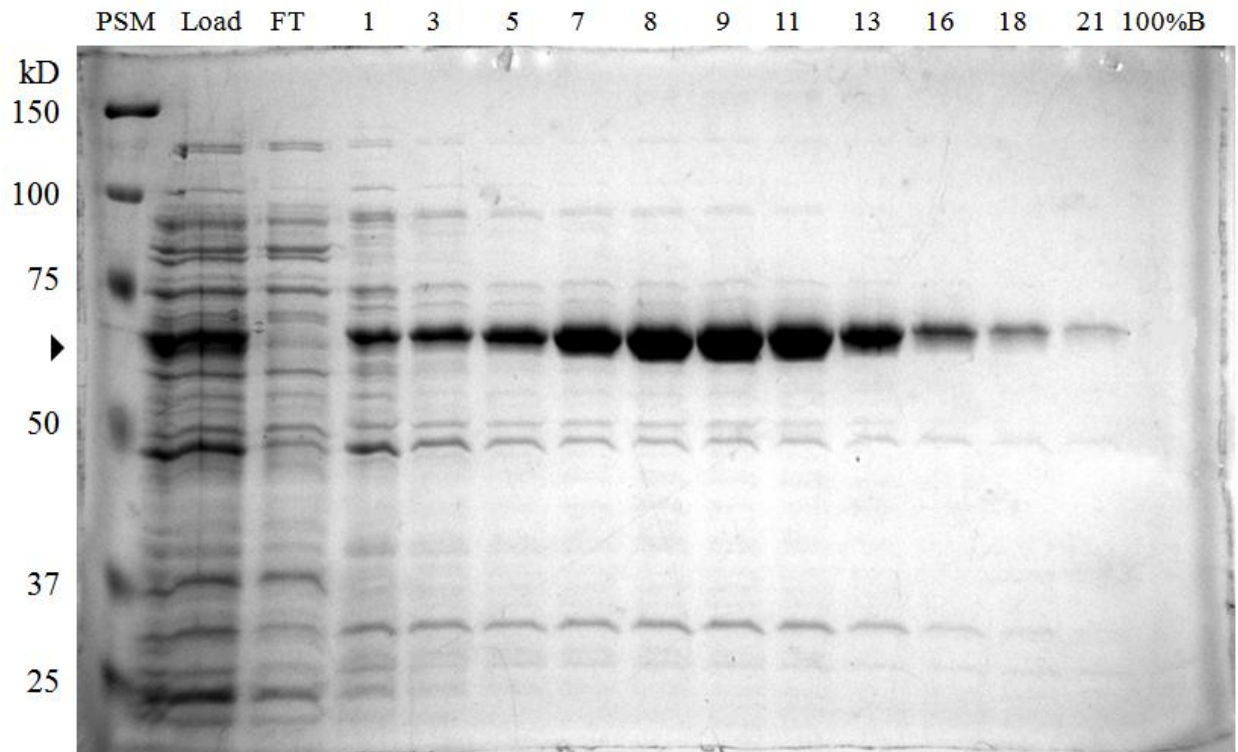

Figure 8 - SDS-Page - Coomassie stain of FASTKD4-6XHis purification

Figure 8: Selected fractions of Ni$^+$ affinity purified total soluble cellular lysate from bacterial cells induced 14 hours at 16°C, analyzed by SDS-Page on 10% polyacrylamide gel and stained with Coomassie blue.

Test for RNA and DNA in affinity purified FASTKD4-3FH

   To test for the presence of RNA and DNA in the affinity purification elution fractions, 10

µl of fraction 8 from the nickel column affinity purification of FASTKD4-6XHis were loaded on

a 1.0% agarose gel and stained with ethidium bromide. No DNA or RNA was apparently visible.

However this test is not conclusive. Because the protein was expressed in *E. coli*, and not in its

native mammalian mouse cell environment, the putative RNA binding RAP domain of

FASTKD4 may not have encountered an appropriate RNA or DNA with which to bind. RNA

and DNA binding assays from mammalian expressed FASTKD4 will be necessary to fully

appreciate possible RNA and DNA that binds FASTKD4.

Plasmids pGS-FASTKD4-EOS and pGS-FASTKD4-3FH

      To generate a stable mammalian cell line with inducible expression of the FASTKD4 fusion protein, the FASTKD4 gene was inserted into two vectors which were transfected separately into naïve 3T3-Switch mouse fibroblast cells: pGS-EOS (Figure 9) and pGS-3FH (Figure 11). Both vectors were designed to insert a target protein directly between a mifepristone inducible promoter and specific tags while at the same time conferring antibiotic resistance. The pGS-EOS vector carries genes for resistance to ampicillin and zeocin and is designed to add a c-terminal EOS protein epitope tag to a protein of interest. The EOS protein is a photoactivatable fluorescent protein, which facilitates immunofluorescent visualization within the cell.
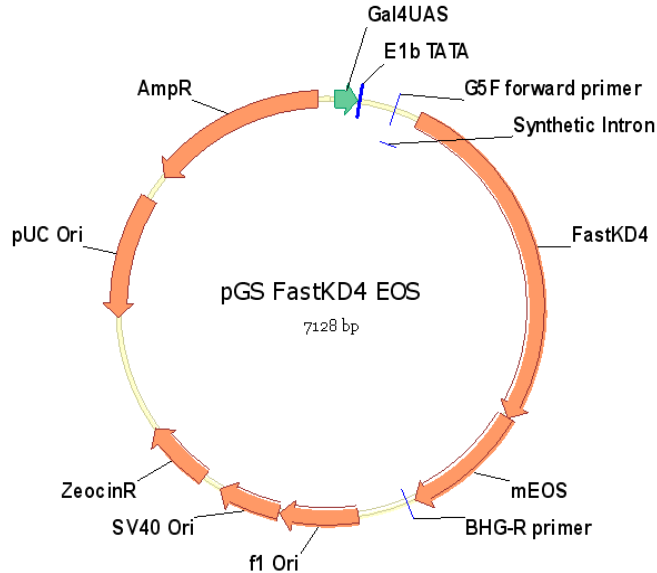

Figure 9 - Mifepristone inducible pGS-FASTKD4-EOS vector

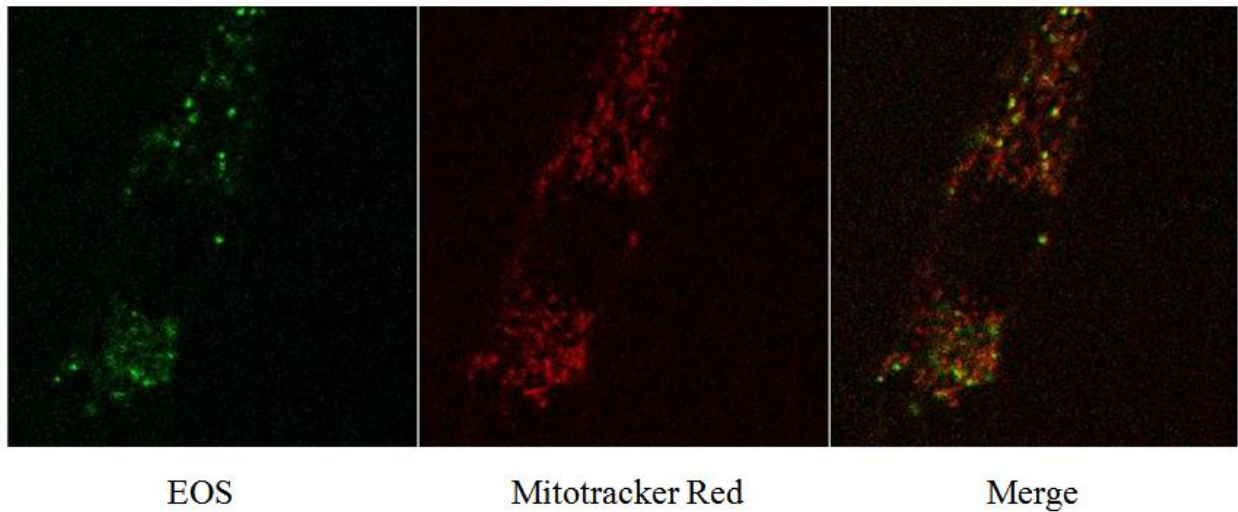EOS   Mitotracker Red   Merge

Figure 10 - Fluorescence microscopy - anti FASTKD4 - EOS

Figure 10:   FASTKD4-EOS localization in 3T3-switch cells. Cells were induced at 37°C for 14 hours with 50 pM mifepristone. Mitotracker Red was applied to a final concentration of 10uM. Cells were fixed and imaged using an excitation wavelength of 543 for Mitotracker red and 488 for EOS.

Immunofluorescent microscopy images of 3T3-switch cells stably transfected with pGS-FASTKD4-EOS reveal that FASTKD4-EOS has both cytoplasmic and mitochondrial localization. In Figure 10 above, mitochondria are indicated by staining with Mitotracker red and the EOS-tagged FASTKD4 protein is indicated by green. Regions of red, green and yellow can be seen in the merged image. Clusters of yellow within the mitochondria suggest that FASTKD4-EOS localizes to distinct sites within the mitochondria, but does not fill the organelle uniformly. However, due to limitations imposed by the resolution and image quality available with our microscope, a clear picture of exactly where FASTKD4-EOS localizes within the cell cannot be determined precisely.
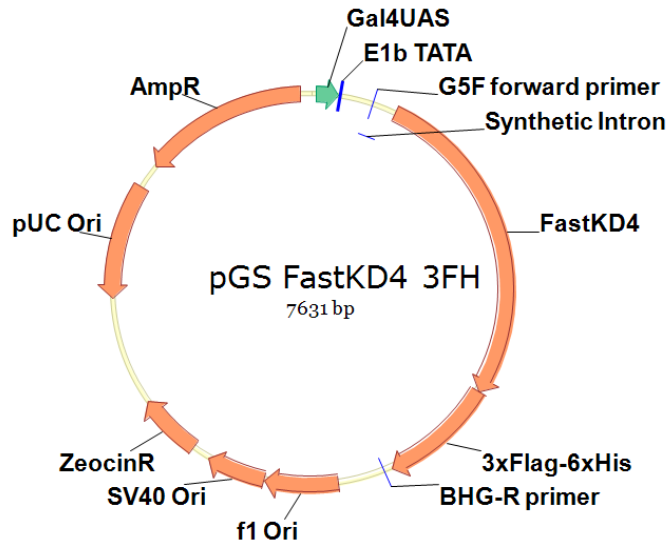
38

Figure 11 - Mifepristone inducible pGS-FASTK4-3FH vector

In addition to carrying genes for ampicillin and zeocin resistance, the pGS-3FH vector inserts target proteins between a mifepristone inducible promoter and two successive epitope tags, 3X FLAG and 6XHis, which allow immunological and affinity-based manipulation of the fusion protein.

Clonal selection of 3T3-FASTKD4-3FH cells

During the transfection process, the manner in which a plasmid containing fusion proteins inserts into the host genome is unpredictable. It may be that the plasmid inserts near a constitutively active promoter, or in multiple copies, a combination of both, or in other problematic ways. To identify cells which have been acceptably transfected, we performed a clonal selection. After the initially transfected cells had stabilized and were growing in medium containing 100% zeocin designated 'Mass Culture,' we selected colonies containing very few cells and established two clonal lines designated 'Clone 1' and 'Clone 2.'

Mifepristone inductions, followed by isolation of total soluble cellular protein, and western blot analyses were performed on each of the cell cultures (Figures 12-14). The western blots were probed with antibodies against the FLAG epitope (Sigma) and the flavoprotein subunit SdhA of Mitochondrial Respiratory Complex II (MitoSciences). Results for Clones 1 and 2 show effective mifepristone induction, without leaky expression of the fusion protein as is evident in Figures 12 and 13 as an absence of the FLAG epitope (71 kD) in the non-induced fractions. However, the mass culture western blot analysis (Figure 12) shows leaky, uncontrolled expression of the FASTKD4-FLAG fusion protein as is evident as a heavy band at 71 kD in the non-induced fraction. The positive control, Complex II is evident in all fractions, as would be expected in an aerobically respiring living mammalian cell. Clone 1 was chosen for use in further experiments due to the very clear absence of the FLAG epitope in the non-induced fraction.
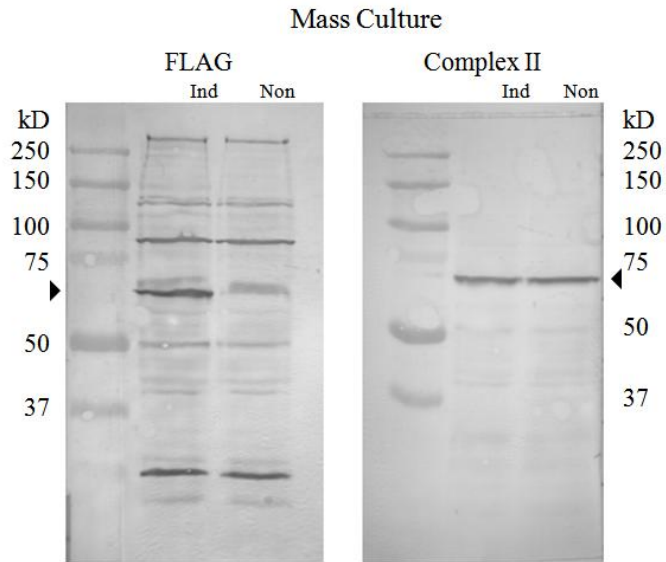
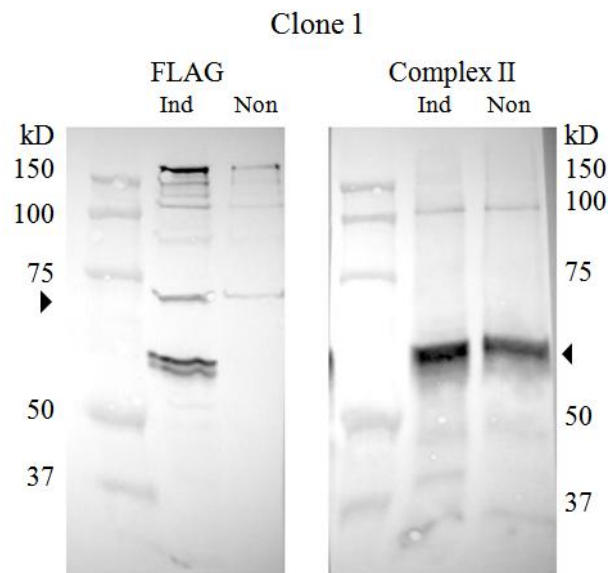**Figure 12 - Western blot analysis - induction test - mass culture**

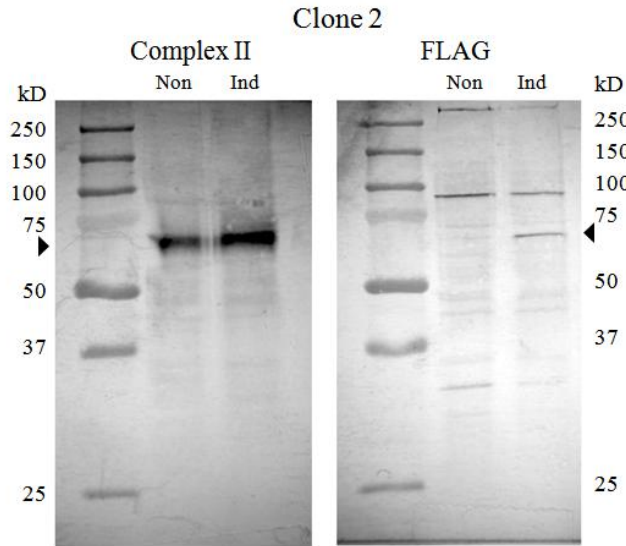**Figure 13 - Western blot analysis - induction test – clone 1**

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Figure 14 - Western blot analysis - induction test – clone 2

Figures 12 – 14: Cells were induced 14 hours at 37°C with 50 pM mifepristone. Total soluble cell lysates from induced and non-induced cells were analyzed by 10 % SDS-page followed by Western blotting. Primary antibodies: Mouse anti-Complex II and rabbit anti-Flag. Secondary antibodies: Phosphatase labeled goat anti-mouse and phosphatase labeled goat anti-rabbit.

Immunofluorescent microscopy of 3T3-Switch FASTKD4-3FH cells

To determine the subcellular localization of the FASTKD4 protein, all three cell cultures were prepared for and viewed with confocal immunofluorescent microscopy. (Figures 15-17) In the mass culture image (Figure 15) it is clear that various cells in the culture express FASTKD4 protein at different levels. The mitochondria of the cell at the top of the image are nearly filled with FASTKD4 and appear to be uniformly yellow. There are small regions of green outside the mitochondria, indicating the presence of FASTKD4 in the cytoplasm. The mitochondria of the cell on the lower portion of the same image do not appear to be uniformly yellow, indicating a lower level of FASTKD4 protein expression. There are distinct regions of red and yellow in the mitochondria with a distribution of green in the cytoplasm. This indicates that while FASTKD4 does reside in mitochondria, it is not exclusively a mitochondrial protein.

The image of Clone 1 (Figure 16) shows a more uniform level of expression of FASTKD4. The pattern of distribution of FASTKD4 within the cell is consistent with observations in the mass culture - it is mitochondrial, but not exclusively, as it is present in distinct regions in both the mitochondria and cytoplasm.

Images of Clone 2 (Figure 17) reveal slight variations in the level of mifepristone induced FASTD4 protein within cells of the same culture. The cell at the left side of the image shows distinct regions of yellow within the mitochondria (red) and distinct points of green in the cytoplasm. This is nearly the same in the cell on the bottom right, except that the mitochondria appear to be more filled with FASTKD4 as the mitochondria show a higher proportion of yellow to red.
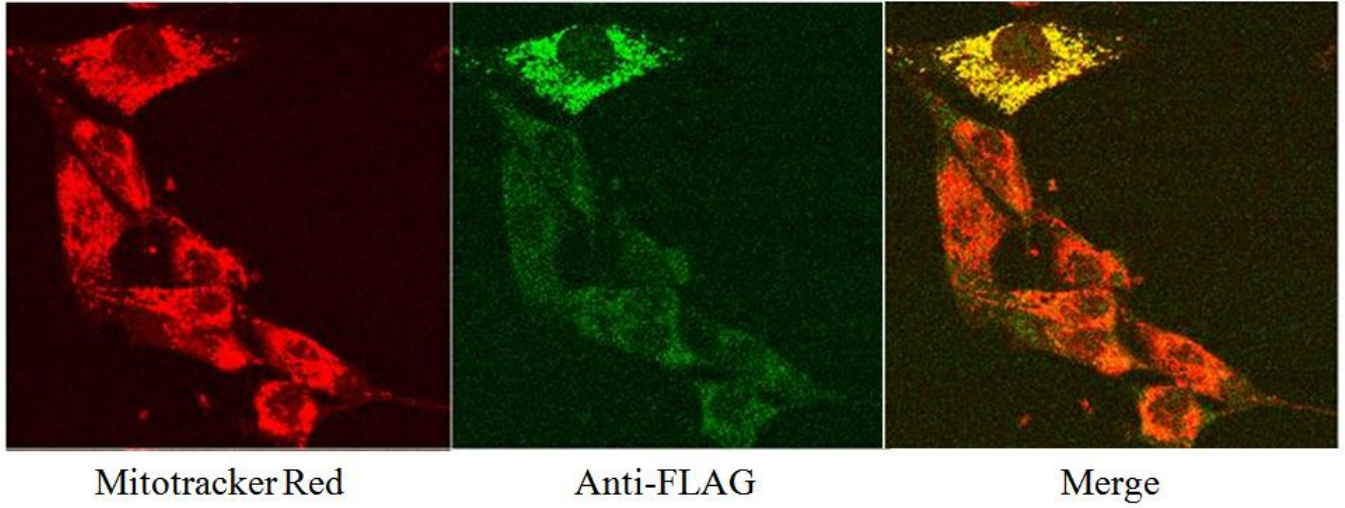
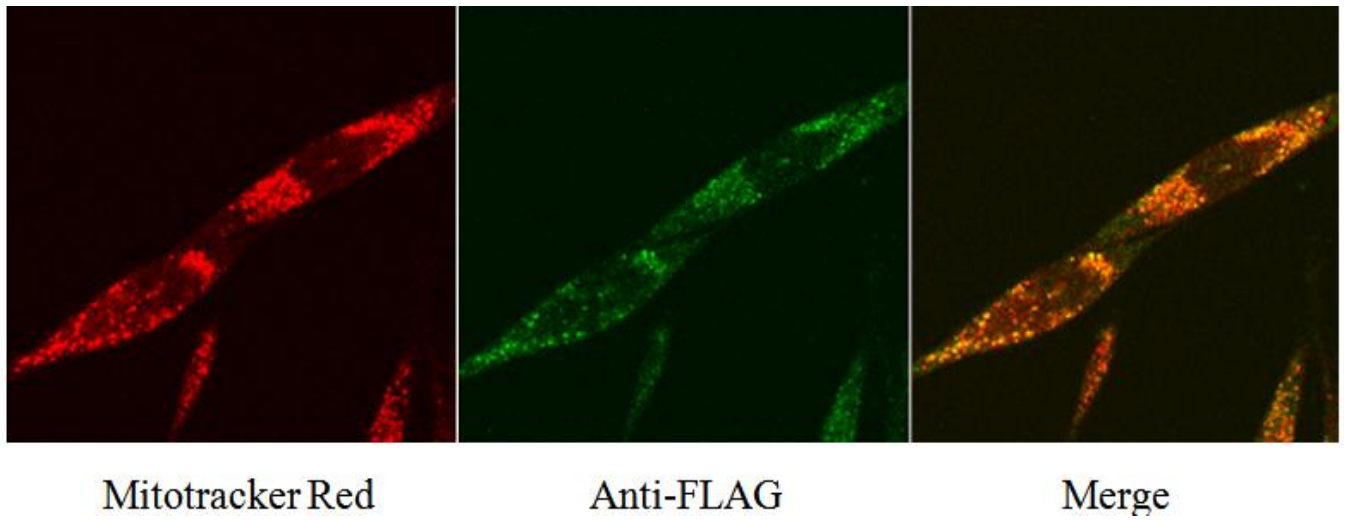**Figure 15 - Immunofluorescence microscopy - Mass Culture**

Figure 16 - Immunofluorescence microscopy – Clone 1

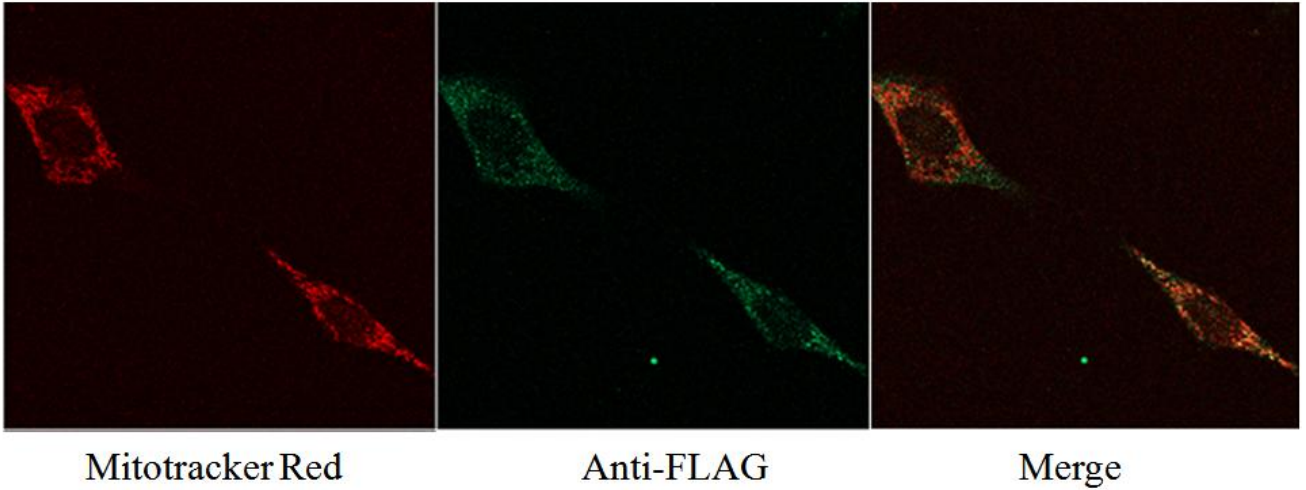Mitotracker Red   Anti-FLAG   Merge

Figure 17 - Immunofluorescence microscopy - Clone 2

Figures 15 - 17: FASTKD4-3FH localization. Cells were induced at 37°C for 14 hours with 50 pM mifepristone. Mitotracker Red was applied to a final concentration of 10uM. Cells were fixed and treated with antibodies. Primary antibody: rabbit anti-Flag 0.05%. Secondary antibody: goat anti-rabbit Alexa-Fluor 488.

Effects of long-term Mifepristone induction

To preliminarily test the overall viability of cells under prolonged mifepristone induced expression of FASTKD-3FH on 3T3 Switch cells, mifepristone was applied to the cell culture and maintained at a final concentration of 50 pM. We observed were no changes in cell proliferation, viability or morphology during the first week of induction. However on the seventh day, the cells stopped proliferating, became enlarged and had irregular morphology. A control, non-induced culture grown in parallel with the induced cells retained normal proliferation, viability and morphology. The results of this suggest that overexpression of tagged FASTKD4 is not acutely toxic, although some toxicity develops with time. Further investigation is necessary to determine the exact cause of the declining health of the cells.

Tandem affinity purification of FASTKD4-3FH from purified mitochondria

To purify FASTKD4-3FH from cell cultures Clone 1 cell culture was expanded and induced for 14 hours with 50 pM mifepristone. Mitochondria were then isolated, purified and lysed and total soluble mitochondrial protein was isolated. FASTKD4-3FH fusion protein was immunoprecipitated from soluble mitochondria protein by incubation of total soluble mitochondrial protein with FlagM agarose beads and eluting with Flag peptide solution. The wash and elution fractions were analyzed by SDS-page on 12% acrylamide gel followed by Western blotting. This analysis shown below (Figure 18, panel A.) suggests that the FASTKD4-3FH may not adhere tightly to the FLAGM beads as bands corresponding to a molecular weight of FASTKD4-3FH (approximately 71 kD) are evident in all wash fractions. This may be a result of the final folded conformation of FASTKD4-3FH which may impede binding to the Flag antibody on the FlagM beads. FASTKD4-3FH eluted well but not completely, as there is evidence of the protein in the fourth and fifth elution fractions.

To detect the presence of possible protein binding partners of FASTKD4-3FH, wash fractions 4 - 5 and elution fractions 1 - 3 were further analyzed by SDS-page followed by silver staining (Figure 18, panel B)

The silver stain analysis of FlagM immunoprecipitation of FASTKD4-3FH reflects the results of the preceding Western blot analysis in that the fusion protein, presenting as a band in the gel corresponding to molecular weight of approximately 71 kD, is present in small amounts in the wash fractions and appropriately larger amounts in the elution fractions. Very faint bands in the silver stained gel lane of the first elution fraction appear at positions corresponding to both higher and lower molecular weights than FASTKD4. This may indicate that the overexpressed FASTKD4-3FH fusion protein does not appear to have significant protein binding partners in ratios approaching 1:1 or even 1:5. One explanation for this might be that FASTKD4 is not part of a large complex, but may interact with its partners transiently or under specific conditions which were not present in the cell culture at the time the cells were harvested. Since FASTKD4 possesses a domain similar to a BH3-like domain, it may be that FASTKD4 might become more interactive with its protein partners during apoptosis or other cellular conditions.
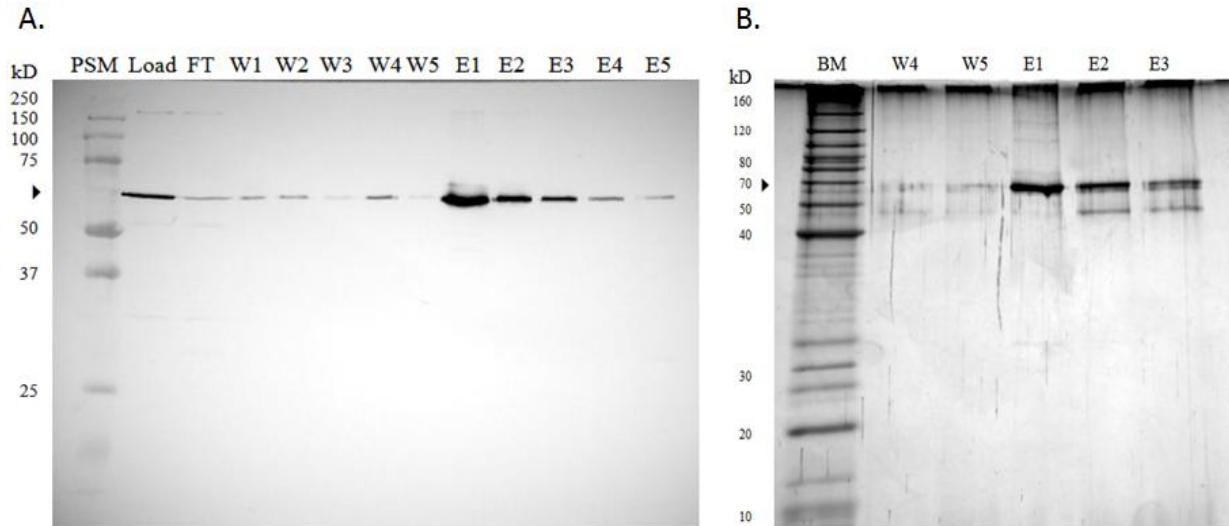
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Figure 18 - Western blot - silver stain of FlagM purification of FASTKD4-3FH

Figure 18: A. Cells were induced with 50 pM mifepristone for 14 hours at 37°C. Total soluble mitochondrial protein was isolated and incubated on FlagM beads. Beads were washed five times with buffer containing .02% TX-100, eluted five times with buffer containing 0.02% TX-100 and 150 ng/ul Flag peptide and analyzed by SDS-Page followed by western blotting. Primary antibody: rabbit anti-Flag. Secondary antibody: phosphatase labeled goat anti-rabbit. B. Wash fractions 4-5, and elution fractions 1-3 from FlagM immunoprecipitation of FASTKD3-3FH were analyzed by 12% SDS-page gel followed by silver staining. BM indicates the BenchMark (Invitrogen) protein ladder here and in other figures.

To further clarify the presence or absence of protein binding partners for FASTKD4, we performed a second purification using Nickel resin beads. FlagM elution fractions 1 - 3 were incubated with nickel resin beads and eluted with 1M imidazole, and analyzed by SDS-page followed by Western blotting (Figure 19, panel A).

The results of the Western blot analysis indicate that the FASTKD4-3FH fusion protein adheres well to the nickel resin beads, as there is no significant presence of it in the wash fractions. A heavy band at 71 kD in the first elution fraction and lighter bands in each successive elution indicate that FASTKD4-3FH eluted well and its presence was significantly reduced by each elution. To identify the presence of possible protein binding partners, the wash fractions and elution fractions were further analyzed by 12% SDS-page, followed by silver staining (Figure 19, panel B).
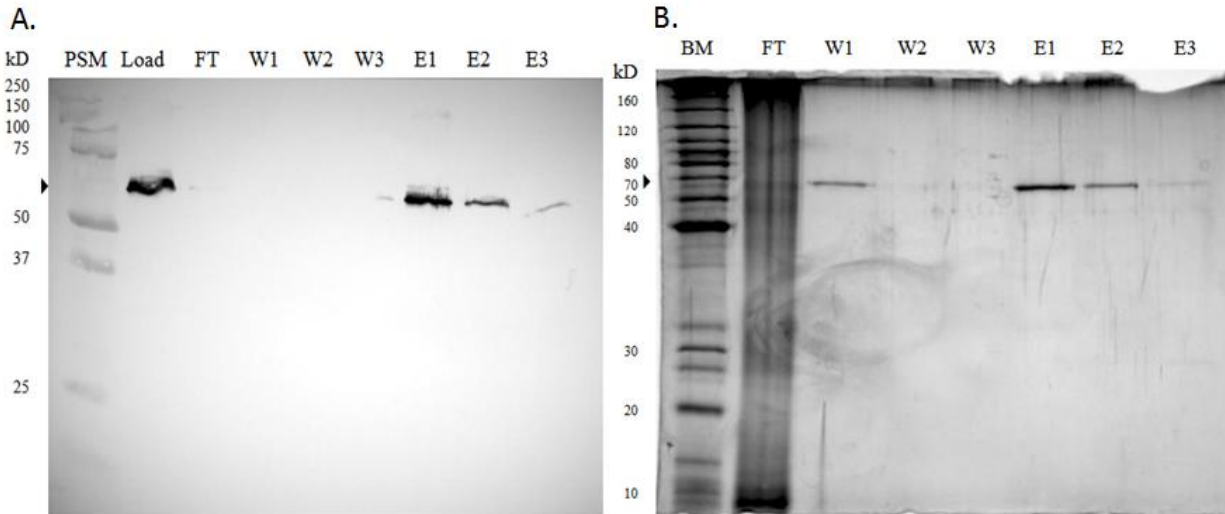
Figure 19 - Western blot – FlagM and Ni$^+$ affinity purification of FASTKD4-3FH

Figure 19: A. FlagM immunoprecipitation elution fractions 1-3 were pooled and 2-mercaptoethanol was added to a final concentration of 2 mM, and the mixture was incubated on Ni$^+$ resin beads for two hours at 4°C. Ni$^+$ resin beads were then washed three times with buffer containing 2.0 mM 2-mercaptoethanol, eluted three times with buffer containing 1M imidazole and 2.0 mM 2-mercaptoethanol, and analyzed by 12% SDS-Page followed by western blotting. Primary antibody: rabbit anti-Flag. Secondary antibody: phosphatase labeled goat anti-rabbit. B. FlagM immunoprecipitation elution fractions 1-3 were pooled and 2-mercaptoethanol was added to a final concentration of 2.0 mM. The mixture was incubated on Ni$^+$ resin beads for two hours at 4°C. The Ni$^+$ resin beads were then washed three times with buffer containing 2.0 mM 2-mercaptoethanol, eluted three times with buffer containing 1M imidazole and 2.0 mM 2-mercaptoethanol, and analyzed by 12% SDS-page followed by silver staining.

The results of this silver stain analysis reflect the results of the western blot analysis in

that there is a very slight presence of FASTKD4-3FH in the wash fractions, a large amount in the

first elution fraction, and sharply diminished amounts in subsequent elutions. There are no

detectable bands representing binding partners of FASTKD4-3FG in the stained gel. This further

supports the results of the initial silver stain analysis of the FlagM immunoprecipitation which

showed very little indication of significant protein binding partners for FASTKD4-3FH.

Summary

Our lab has begun to identify the role and function of FASTKD4 in the mitochondria. To this end, we have established stable cell lines expressing the epitope-tagged FASTKD4 protein. We have confirmed the localization of FASTKD4's within the mitochondria and immunofluorescent imaging has revealed that FASTKD4 also resides in the cytosol. Sequence alignment has identified a BH3 like domain in FASTKD4. Further testing may determine if this domain allows FASTKD4 to play a role in apoptosis. Two putative transmembrane domains within FASTKD4 suggest that it may be anchored in mitochondrial membrane. Compartmental mitochondrial analysis and sodium carbonate extraction will be useful in clarifying the precise location and orientation of FASTKD4. Sustained mifepristone induced overexpression of FASTKD4-3FH has not proven to be acutely toxic to cells. FlagM immunoprecipitation reveals that FASTKD4-3FH does not bind tightly to Flag antibodies as many other proteins do. It is possible that the c-terminal Flag epitope is prevented from binding because it may reside within the final folded form of the protein, or that it is partially obscured by other domains or binding partners or is a site of oligomerization. Tandem immune/affinity purification analysis has preliminarily revealed that FASTKD4 does not appear to closely associate with significant quantities of other proteins. To determine if FASTKD4 interacts with other proteins via its BH3-like domain during apoptosis, further tests such as tandem purifications after the onset of apoptosis or other cellular stresses could be performed. It is possible that FASTKD4 may function as a dimer or an oligomer in its active form. Protein cross-linking, followed by SDS-page and western blotting in the absence of reducing agents may help to determine this. Since FASTKD4 contains two putative RNA binding domains it would be interesting to identify RNA

binding partners. This could be attempted by immunoprecipitating FASTKD4, performing an

RNA extraction which could then be subjected to RT-PCR.

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