Identification and Characterization of TMEM 85, a Novel Suppressor of Bax-mediated Cell Death in Yeast

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Your file Votre référence
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To my family,

For all their love, help and support.

Abstract

The ability to evade apoptosis is an acquired characteristic associated with many normal and pathophysiological processes. TMEM 85 represents a novel transmembrane domain containing human protein isolated in our previous screen for Bax suppressors, but whose function is currently unknown. Using viability and growth assays, we confirmed that TMEM 85 is anti-apoptotic. Four unique human cDNA sequences containing regions distinct from and of perfect identity to our cDNA were present in the database. Analysis of *TMEM 85* suggests that it consists of five exons, alternatively spliced to produce at least four different mRNA's and proteins (TMEM 85v1-v4). RT-PCR analysis using RNA isolated from mice and humane tissues show that all transcripts are expressed. Yeast contain an orthologue of the human TMEM 85v1 protein, YGL213C. Surprisingly, the viability assay indicated that mutants lacking YGL231c do not show a hyper-responsive apoptotic phenotype, however its overexpression shows that it is nevertheless anti-apoptotic. Using a yeast strain expressing chromosomally TAP-tagged YGL231c, we found no up-regulation of the endogenous gene due to stress. The deletion mutant is also known to expresses a synthetically lethal phenotype in the presence of α-synuclein. While expression of α-synuclein caused significant death in both the wild type and deletion mutants, TMEM 85v2 was unable to exhibit a protective role. These findings demonstrate the complexity of the TMEM 85 gene and its anti-apoptotic function in both yeast and human.

Résumé

La capacité d'échapper à l'apoptose est une caractéristique acquise par la cellule et associée à de nombreux processus autant normaux que pathophysiologiques. En passant au crible des librairies d'expression d'ADN complémentaires chez la levure, nous avions auparavant identifié, dans notre laboratoire, de nouvelles séquences anti-apoptotiques qui, lorsque isolées, ont été capables d'inhiber les effets mortels de l'expression hétérologue de la protéine Bax des mammifères. Nous signalons, dans ce rapport, l'identification et la caractérisation d'un des inhibiteurs de Bax, TMEM85. A travers un système de mesure de viabilité chez la levure et l'expérience d'Halo, nous avons confirmé que la surexpression de TMEM85 peut réduire les effets apoptotiques du peroxyde d'hydrogène. La comparaison de notre séquence d'ADN complémentaire avec celles disponibles dans la base de données de NCBI, a indiqué que notre clone contient une extrémité 3' unique, suggérant qu'un épissage alternatif pourrait créer plusieurs transcripts. Quatre séquences d'ADN complémentaires uniques des humains exhibant un fort degré de similarité avec la notre étaient présentes dans la base de données. Les analyses des séquences ainsi que celle du génome humain qui y est associée ont révélé que le gène TMEM85 est composé de cinq différents exons épissés alternativement pour produire au moins quatre différents ARN messagers et protéines (TMEM 85v1, TMEM 85v2, TMEM 85v3 et TMEM 85v4). Le gène s'étend sur une distance de plus de 5.1 Kb sur le chromosome XV humain. En utilisant l'ARN isolé de divers tissus de souris et de lignées cellulaires humaines, la réaction en chaîne de Transcriptase Polymérase renversée (RT-PCR) a montré que tous les transcripts sont exprimés. Le transcript TMEM 85v1 augmenta suite aux effets apoptotiques du facteur nécrosant des tumeurs (TNF-α). Un orthologue de la protéine humaine TMEM 85v1, YGL213C, est exprimée chez la levure et se localise dans le réticulum endoplasmique. Le rôle de cette protéine fut évalué en utilisant une levure mutante, dépourvue de YGL231C (YGL231cΔ). Le système de mesure de viabilité indiquât que les mutants dépourvus de ce gène ne

démontrèrent aucun phénotype apoptotique accru. Cependant, la surexpression de YGL231c eut pour conséquence de protéger la levure contre l'apoptose provoquée par le peroxyde d'hydrogène. Quoique YGL231c pourrait avoir un rôle anti-apoptotique, ces résultats suggèrent que la levure exprime des protéines compensatoires ayant une fonction similaire. Nous avons également découvert que TMEM 85v2 fut capable de suppléer au manque de YGL231c dans les mutants. Par conséquent, sa fonction anti-apoptotique pourrait être indépendante de TMEM 85v1. En utilisant une souche de levure exprimant le gène YGL231c modifié au niveau du chromosome par l'addition de l'étiquette TAP, l'analyse western n'indiquât aucune augmentation dans la régulation du gène endogène suite à des conditions de stress induites par le peroxyde d'hydrogène. Le mutant présente également un phénotype malade en présence de α-synuclein, un inducteur du stress du réticulum endoplasmique. Quoique l'expression de α-synuclein causât la mort des souches normales aussi bien que mutantes, TMEM 85v2 a été incapable de manifester un rôle protecteur. Ces résultats démontrent la complexité du gène de TMEM 85 et sa fonction anti-apoptotique autant chez la levure que chez l'humain.

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I have completed and organized all the experiments and results in this thesis, with the following exceptions. The Bax screen on the cDNA library and subsequent isolation of the TMEM 85 cDNA was performed by Chamel Khoury. RT-PCR of both human and mouse tissue, excepting design of the primers, was completed by Zhao Yang.

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1.1 Apoptosis

1.1.1 Introduction

Apoptosis, a form of programmed cell death first described in 1972 (Kerr, et al. 1972), has been well characterized in metazoan cells. This active cellular process commits cells to a death pathway independent of necrosis-essentially committing suicide (Fink and Cookson 2005). Apoptotic cells exhibit characteristic morphologic and genetic changes which are distinct from necrosis and result in disassembly of the cell without associated inflammation and pathology. Apoptosis is an essential process for both the development and long term survival of multicellular organisms. The process of apoptosis performs many roles in multicellular organisms: homeostasis, embryogenesis and organ development, and removal of infected or irreparable cells (Lawen 2003). Deregulation of the apoptotic process is one of the underlying causes of cellular pathologies leading to disease including heart disease (Morissette and Rosenzweig 2005; Kutuk and Basaga 2006), cancer (Vermeulen, et al. 2005), autoimmune (Navratil, et al. 2006) and neurodegenerative disorders (Krantic, et al. 2007), as well as many others (Fadeel, et al. 1999).

Apoptosis displays characteristic morphological features: rounding of the cell and pyknosis, condensation of nuclear DNA, fragmentation of the nucleus, and formation of membrane bound vesicles termed apoptotic bodies (Kroemer, et al. 2005). There are secondary features associated with apoptosis which may be

used, according to Kroemer et al., to diagnose but not define the process. These features include activation of caspases, typically the fundamental mediators of apoptosis, uncovering of apoptotic specific cell surface molecules such as phosphatidylserine, and fragmentation of nuclear DNA. None of these secondary features can be categorically stated to be required for the development of the apoptotic phenotype, though typically all are associated with apoptosis. Apoptosis is unique from other forms of cellular death and is often placed in direct contrast to necrosis which is induced by external, pathologic stimuli, and presents morphologically as oncosis. The final stage of necrosis, cell lysis with associated inflammatory responses, can be viewed as its defining characteristic (Hail, et al. 2006).

There are three loosely identifiable stages of apoptosis, each with specific regulators: initiation, effector, and execution (Green and Kroemer 1998). The factors involved in initiation depend on the type of cell and the type of external apoptotic stimuli being received by the cell (Hail, et al. 2006). Initiations factors, which are responsible for collecting and integrating external and internal signals, determine if a cell proceeds into apoptosis. During the effector stage, effector molecules become activated, and in most cases an irreversible cascade towards death begins. The final stage of apoptosis, execution, involves the fragmentation of nuclear DNA and phagocytosis of the apoptotic bodies.

1.1.2 A Brief History of the Genetics of Apoptosis

The controlled nature of apoptosis led to the belief that the underlying process was genetic in nature. It was known that *Caenorhabditis elegans* underwent a programmed death in a predicable fashion of its healthy somatic cells during development (Sulston and Horvitz 1977; Sulston, et al. 1983) and it was in these nematodes that the genetics behind this cellular suicide was first investigated (Sulston, et al. 1983; Ellise and Horvitz 1986). Through these studies the first pro-apoptotic genes were identified, the most important for understanding the conserved process of apoptosis being *ced-9*, *egl-1*, *ced-4*, and *ced-3* (Ellise and Horvitz 1986). Each of these was later found to have mammalian homologues which are vital for regulating apoptosis. Both *ced-9* and *egl-1* are members of the Bcl-2 family of apoptotic regulators. The anti-apoptotic CED-9 and its mammalian homologue Bcl-2 (Lettre and Hengartner 2006) work antagonistically with EGL-1 and its homologue, a BH3 domain only protein, as regulators of initiation of apoptosis (Hengartner and Horvitz 1994). This balanced relationship is typical of the Bcl-2 family which includes anti-apoptotic Bcl-2, Bcl-X_L and Bid, and pro-apoptotic Bax and Bak (Conradt and Horvitz 1998). An adaptor protein CED-4 and its mammalian homologue APAF1 (Zinkel, et al. 2006), mediate the final step in activating the caspase cascade which induces the effector stage of apoptosis. The mammalian homologue of *ced-3* encodes for Interleukin1β converting enzyme (Zou, et al. 1997). This protease was found to be part of a larger family, termed the caspase family (cysteine-dependent aspartate specific

proteases), which are now recognized as the main effectors of apoptosis (Rupinder, et al. 2007).

1.2 Caspases

1.2.1 Defining Initiator and Effector Caspases

There are two groups of caspases involved in apoptosis, initiators and effectors. There is also a third group of caspases not involved in apoptosis that serve a critical role in inflammatory responses, which will not be covered in this work (Dinarello 1998; Cornelis, et al. 2007). Initiator caspases are activated due to upstream signalling events resulting from the integration of external or internal death signals (Riedl and Shi 2004). They have large pro-domains which are responsible for protein-protein interactions between initiators and their adaptors (Rupinder, et al. 2007). Effector caspases are activated by initiators and have much smaller pro-domains. Both types of caspases are constitutively produced but exist as enzymatically inactive zymogens (Hail, et al. 2006).

1.2.2 Caspases can be Activated by Extrinsic and Intrinsic Apoptotic Pathways

Apoptosis can be induced via the extrinsic or intrinsic pathway, each with its own set of specific activation and signal transduction pathways. The extrinsic pathway occurs as a result of integration of extracellular signals (death ligands) from proteins such as FasL or TRAIL (Nagata 1999; Wiley, et al. 1995). These signals result in death receptor clustering and recruitment of the initiator caspases specific to the extrinsic pathway, caspase-8 and caspase-10 (Muzio, et al. 1996; Kischkel,

et al. 2001). The specific method of activation of these initiator caspases remains unclear; however the death inducing signalling complex (DISC) remains a good model (Riedl and Salvesen 2007). Once activated the initiator caspases are responsible for both the proteolytic cleavage and activation of effector caspases, as well as initiation of mitochondrial apoptotic signalling (Degli Esposti 2004).

The intrinsic pathway is mediated via the release of apoptotic signalling proteins from the mitochondria. Release of these proteins results in the formation of the apoptosome. Control of release is largely determined by the balance between members of the Bcl-2 family which reside in the mitochondrial membrane during apoptosis, the best characterized being Bax and Bcl-2 (Reed, et al. 1998). The apoptosome complex mediates the activation of another initiator caspase, caspase-9 (Acehan, et al. 2002). Although caspases are mostly thought to act downstream of the mitochondria, exceptions do exist. For example, the role of caspase-2 has not yet been fully defined. It is known that caspase-2 is activated via mitochondrial induced apoptosis, but some reports suggest it plays a role in Bax translocation resulting in the release of the apoptotic signalling proteins cytochrome c and DIABLO from the mitochondria (Lassus, et al. 2002), and has the ability to release cytochrome c independently (Guo, et al. 2002); conflicting reports show that caspase-2 requires the apoptosome for activity and is therefore downstream of Bax (Samraj, et al. 2007).

After initiator activation, both the intrinsic and extrinsic pathways converge on effector caspase-3, caspase-6, and caspase-7 (Sprick and Walczak 2004). Once these are activated the cell is usually committed to the apoptotic pathway. The

activated effector caspases are responsible for the typical apoptotic characteristics seen in mammalian cells including pyknosis and nuclear fragmentation (Buendia, et al. 1999), formation of apoptotic bodies (Rudel and Bokoch 1997), activation of a nuclease that mediates DNA fragmentation (Enari, et al. 1998) and externalization of phosphatidylserine (Balasubramanian, et al. 2007).

1.3 Bcl-2 Family: Regulators of Apoptosis

The Bcl-2 family of proteins are largely responsible for regulating the release of pro-apoptotic proteins from the mitochondria including cytochrome c (Kluck, et al. 1997), Smac/DIABLO (Du, et al. 2000), Omni/HtrA2 (Suzuki, et al. 2001), EndoG (Li, Luo, and Wang 2001) and AIF (Susin, et al.). They can be broadly classified into two groups, anti- or pro-apoptotic. This family is characterized by the presence of conserved sequence motifs, termed Bcl-2 homology domains (BH), of which there are four. Substituent members can carry any combination of the BH domains, though typically the anti-apoptotic members contain BH1-4, while the pro-apoptotic members lack BH4 (Chan and Yu 2004). The best characterized pro-apoptotic members, Bax and Bak, reside in the outer mitochondrial membrane (OMM) and cytosol respectively, during non-apoptotic conditions. Once an appropriate apoptotic signal is received, pro-apoptotic members undergo a conformation change (Chan and Yu 2004; Griffiths, et al. 1999), and in the case of Bax, translocate to the OMM (Wolter, et al. 1997). There, they oligomerize and form pores in the OMM, making it permeable to pro-apoptotic compounds stored in the mitochondria (Antinssin, et al. 2000). The anti-apoptotic members serve as a check for inducing apoptosis; members, including

Bcl-2 and Bcl-X_L, heterodimerize with pro-apoptotic members of the family (Ruffolo and Shore 2003). This prevents the formation of pores in the outer mitochondrial membrane thereby protecting the cell from death. Anti-apoptotic proteins may also prevent cell death in a Bax independent manner (Callus and Vaux 2007).

1.4 Alternative Forms of Programmed Cell Death

While caspase induced apoptosis remains the best characterized form of programmed cell death, other alternative forms exist. Caspase independent cell death can occur when apoptotic inducing molecules are released from the mitochondria, inducing cell death but bypassing caspases as the effector proteins (Green and Kroemer 1998). These death inducing proteins AIF, EndoG, and HrtA1/Omi can be mediators of both a caspase dependent and independent pathway, and are released by the Bcl-2 family (Orrenius, et al. 2007).

Autophagy is a process by which cellular constituents are utilized to enhance survival during periods of nutrient deprivation. Prolonged periods of autophagy lead to the destruction of the cell through internal enzymatic degradation without chromosome condensation (Kroemer, et al. 2005). Cellular components are engulfed in autophagosomes, membrane bound vesicles that fuse to lysosomes; the components are then degraded (Klionsky and Emr 2000). The process of autophagy is also involved in tissue development, differentiation and remodelling (Levine and Klionsky 2004). Autophagy may also trigger the cell death pathway involved in apoptosis (Kroemer, et al. 2005).

1.5 Indentifying Novel Sequences involved in Apoptosis

Many methods of identifying genes involved in apoptosis have been utilized: microarrays (Fleischer, et al. 2007), subtractive hybridization (Zhang, et al. 2006), and yeast two hybrid screens (Kim, et al. 2006). These methodologies have revealed a variety of proteins involved in apoptosis from common inhibitors such as heat shock proteins or members of the Bcl-2 family to orphan genes whose role in apoptosis has not yet been characterized. Utilizing the knowledge that Bax induces apoptotic death in yeast, screens of mammalian genes have been undertaken by numerous groups to identify anti-Bax, and therefore possible anti-apoptotic genes (Xu and Reed 1998; Yang, et al. 2006; Osborn and Miller 2007).

1.6 Alternative Splicing

1.6.1 Alternative Splicing Increases Functional Protein Diversity

Since the publication of the nucleotide sequence of the human genome, the important role of post-translational modification in the generation of protein diversity has become increasingly apparent (Graveley 2001). Of these, splicing of pre-mRNA from a single gene to generate several transcripts is arguably the most crucial. Alternative splicing is a key process in creating the complex protein profiles seen in humans. Alternatively spliced transcripts produce proteins that typically have different or antagonistic properties (Hardy and O'Neill 2004; Yang, et al. 2007). This allows cells to maintain a high degree of control in the regulation of cellular processes without needing an equally large genome. It is a necessary process in regulating development and tissue diversity; recent analysis

of the genome indicates that more than 70% of human genes may be alternatively spliced (Johnson, et al. 2003).

Mammalian genes exist as discontinuous sequences of exons (coding regions), that are separated by introns (noncoding regions) that are transcribed together to produce pre-mRNA. Genes, on average, consist of eight exons and a variable number of introns that are typically longer than exons (Pajares, et al. 2007). Once the gene has been transcribed, there are two main forms of splicing to remove noncoding introns: constitutive (the pre-mRNA is always spliced the same way giving rise to one isoform) and alternative (multiple splice sites exist to produce several transcripts). The basic mechanism of alternative splicing involves the recognition of a splice site by a complex called a spliceosome. The spliceosome, consisting of five small nuclear ribonucleoproteins (snRNP) and numerous other factors, acts in the nucleus on pre-mRNA by recognizing a splice site (Black 2003). Through a variety of RNA-RNA, RNA-protein and protein-protein interactions, the introns and unwanted exons are removed, while the remaining exons are joined together (Baralle and Baralle 2005).

In typical splicing events, mediated by the U2 dependent spliceosome, introns and exons are defined by flanking consensus sequences (AG and GU) which are the primary indicators for both constitutive and alternative splicing (Senapathy, et al. 1990). Also important for the recognition of splice sites by the spliceosome are two variable regions known as the branchpoint and the polypyrimidine tract. However, alternative splice sites are generally weaker than constitutive splice sties and often present with atypical compositions (Thanaraj and Stamm 2003).

Alternative splice sites have intron-exon consensus sequences with a high degree of variability; consequently the spliceosome relies heavily on other regulatory elements. These regulatory elements are varied and include both cis and trans elements (Black 2003). Cis elements, used by the spliceosome to better isolate intron-exon borders, act as exonic and intronic splicing silencers or exonic and intronic splicing enhancers located within the pre-mRNA sequence. Trans elements play a larger role in determining the proteome of a cell. They are typically binding factors, such as arginine-serine rich(SR) proteins (Crispino, et al. 1994) or heterogeneous nuclear ribonucleoprotein (hnRNP) (Mayeda and Krainer 1992), that bind to silencer or enhancer regions to affect splicing. A minor type of splicing, seen in approximately 1% of splicing events, is mediated by the U12 dependent spliceosome (Levine and Durbin 2001). It recognizes a different and more variable census sequence (AT and variable 3' splice site), has a different and more constrained branching sequence, and lacks a polypyrimidine tract at the 3' end (Hall and Padgett 1994). Both types of spliceosome are expressed concurrently in the cell and a pre-mRNA may be spliced by both types of machinery (Will and Lührmann 2005). In essence, alternative splicing is due to an ever changing affinity between the various regulatory elements and the spliceosome.

1.6.2 Alternative Splicing and Apoptosis

Alternative splicing has been implicated in regulating a wide variety of cellular processes including gender determination (Schütt and Nöthiger 2000), immune responses (Khabar and Young 2007), and apoptosis (Schwerk and Schulze-

Osthoff 2005). Many proteins known to be key regulators of apoptosis are alternatively spliced, regulating changes in their activity. Some alternatively spliced genes, such as *Smac/DIABLO* and *Bid*, produce multiple transcripts that are functionally distinct (Fu, et al. 2003; Renshaw, et al. 2004). Proteins produced by the shorter alternatively spliced transcripts of caspase 2, Casp2S, and caspase 9, Casp9S, inhibit cell death by asserting an antagonistic (Wang, et al. 1994) or dominant negative (Seol and Billiar 1999) effect on their longer variants. Alternative splicing is a necessary regulator of proteins at all levels of apoptotic induction and execution including death receptors (Ayroldi, et al. 1999), adaptor proteins (van Eyndhoven, et al. 1998), Bcl-2 family members (O'Connor, et al. 1998), caspases, and many others. Results from our lab have shown that our previously identified Bax suppressor also represent complex genes that are alternatively spliced to produce structurally and functionally distinct proteins (Yang, et al. 2006; Khoury, et al. 2007).

1.7 *Saccharomyces cerevisiae* as a Model for Apoptosis

1.7.1 Yeast as a Model System

In ideal processes circumstances, investigation of cellular or molecular biology would be performed *in vivo* in the cells and under whatever conditions the protein or gene was originally identified. This is almost always impossible. Cost, time, control of the environment, even the skills necessary to manipulate the cells typically prove too restrictive to result in meaningful research. Alternative and equivalent model systems are often created to compensate. Mice are used as *in*

vivo models of human disease and for determining pharmaceutical safety (Houdebine 2007), invertebrates are used to screen for novel drugs (Ségalat 2007), while cultured cell lines are used to elucidate processes occurring in complex metazoan cells (Vincan, et al. 2007).

The yeast *Saccharomyces cerevisiae* is one of the best characterized and understood eukaryotic microorganisms, therefore making it an ideal model for basic cellular and molecular research (Mager and Winderickx 2005). Elucidation of its genome, finished in 1996, made it the first eukaryotic genome to be completed (Goffeau, et al. 1996; Mewes, et al. 1997). A number of genetically modified yeast have been developed and are made available to researchers for a minimal cost. Deletion mutants have been created for every yeast gene and are easily available from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf) or the Saccharomyces Genome Deletion Project (http://www-sequence.stanford.edu/group/yeast_deletion_project). A GFP or chromosomally TAP-tagged version of most of the yeast genes currently exists allowing for easy studies of regulation and interaction profiles (Chaves, et al. 1997; Rigaut, et al. 1999). There is extensive knowledge surrounding *S. cerevisiae* which is easily accessible through the internet including genome/proteome databases with related literature (http://www.yeastgenome.org), homology search engines (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=homologene), databases of yeast homologues to human disease genes (http://mips.gsf.de/proj/yeast/reviews/human_diseases.html), localization databases (http://yeastgfp.ucsf.edu), expression profile databases (http://genome-

www5.stanford.edu), and morphological databases (http://scmd.gi.k.u-tokyo.ac.jp/datamine). As fungi, it undergoes a high degree of homologous recombination, creating a system in which exogenous DNA can be introduced in a systematic and highly controllable manner (Fincham 1989). Yeast is relatively cheap to purchase and maintain, grow and replicate in a short time, and is easy and safe to handle. As well as providing a machine in which to study human genes, knowledge about yeast genes has begun to be applied to their human homologues (Osborn and Miller 2007). Homologues often share similar characteristics and provide a good initial insight into the function and regulation of human genes (Ben Yehuda, et al. 1998).

1.7.2 *S. cerevisiae* Undergoes Apoptosis

Until recently, it was a highly debated issue of whether yeast underwent apoptosis as seen in metazoan cells (Fraser and James 1998). The evolutionary role of having such a biochemical pathway could not be rationalized. Apoptosis in a multicellular organism serves the organism as a whole, while in a single cellular organism, does nothing but result in death. It is not immediately apparent how containing an underlying and conserved mechanism of programmed cell death would be a good survival trait for a single celled organism. It is now being argued that in some cases communities of microorganisms act in a manner similar to a multicellular organism, as committing suicide grants an evolutionary advantage on the population (Büttner, et al. 2006). Yeast clearly illustrates the role apoptosis plays for microorganisms (Palková and Váchová 2006). Cell death induced by mating factor occurs when yeast are unable to mate (Severin and

Hyman 2002). This favours evolutionary diversity and a diploid state, both which serve adaptive advantages. Under conditions of nutrient deprivation or hardship, aged yeast commit suicide (Laun, et al. 2001), thereby preserving nutrients for younger and fitter generations.

It has been argued that this death is not actually a form of apoptosis, at least not the apoptosis that is seen in metazoan cells. Extensive studies of the past ten years conclusively show that *S. cerevisiae* does undergo a form of intrinsic apoptosis comparable to that seen to multicellular organisms (Madeo, et al. 1997; Ligr, et al. 1998; Yamaki, et al. 2001; Laun, et al. 2001; Del Carratore, et al. 2002; Madeo, et al. 2002; Eisler, et al. 2004; Silva, et al. 2005). Yeast showing an apoptotic phenotype of chromatin condensation, DNA degradation, and externalization of phosphatidylserine were first identified in 1997. The phenotype was due to a mutation in a gene whose human homologue was later revealed to serve an anti-apoptotic function (Shirogane, et al. 1999). Many homologues to human genes known to be involved in the apoptotic pathway have been found in yeast (Fröhlich, et al. 2007). The human genes *AIF1* (Wissing, et al. 2004), *Htra/Omi* (Fahrenkrog, et al. 2004), and the caspase family (Madeo, et al. 2002), as well as many others, all have yeast homologues. YCA1, the yeast metacaspase, has extensive proteolytic activity toward known caspase substrates, acting as an initiator caspase (Madeo, et al. 2002). Disruption of YCA1 results in increased survival of yeast when exposed to a variety of stresses including increased levels of reactive oxygen species due to exogenously supplied hydrogen peroxide. Other hallmarks of mammalian apoptosis are also seen in yeast under conditions of

stress: cytochrome c is released from the mitochondria (also under heterologous expression of mammalian Bax)(Ludovico, et al. 2002; Priault, et al. 1999), the AIF1 homologue Aif1p is also released from the mitochondria (Wissing, et al. 2004), and yeast present with the characteristic apoptotic morphology (Wolter, et al. 1997).

Yeast has long been used as a model in which to express human genes, both to determine their function and to discover their interacting partners through yeast two hybrid assays. The results of such investigations were the discoveries that mammalian pro-apoptotic proteins Bax (Ligr, et al. 1998), caspases (Kang, et al. 1999), and the Apaf-1 homologue, CED4, induced apoptosis in yeast (Tao, Walke, and Morgan 1999). The human anti-apoptotic genes Bcl-X_L and Bcl-2 were found to protect the yeast from the induced apoptosis (Tao, et al. 1997). This provided a new model system in which genes could be investigated, exploring their anti-apoptotic or pro-apoptotic role. Yeast two hybrid assays using Bax as bait, were the first studies to show mammalian Bax can serve to induce apoptosis in yeast (Sato, et al. 1994).

1.7.3 Inducers and Inhibitors of Yeast Apoptosis

Apoptosis is known to have a variety of inducers which range from specific molecules to general conditions of stress, both external and internal. Generally external factors induce some type of cellular damage from which the cell is unable to recover. Internal inducers of apoptosis are compounds which are produced by the cell as a result of internal damage caused by external stimuli or by faulty

cellular machinery and repair mechanisms. In yeast, low doses of reactive oxygen species (in the form of hydrogen peroxide)(Madeo, et al. 1999), ionic stress (Huh, et al. 2002), UV induced DNA-damage (Del Carratore, et al. 2002), starvation (Eisler, et al. 2004), and N-glycosylation defects leading to ER stress (Hauptmann, et al. 2006) have all been shown to induce the morphologic characteristics associated with apoptosis. The introduction and over expression of heterologous pro-apoptotic genes, such as Bax, also induces apoptosis (Ligr et al. 1998).

Several molecules that act to protect yeast from apoptosis have also been indentified. More general protection is conferred on yeast cells by heat shock and anti-oxidant genes. Utilizing heterologous expression of Bax the proteins HMGB1, Vps24β, and prion protein (PrP^c) were identified as anti-apoptotic in yeast (Brezniceanu, et al. 2003; Khoury, et al. 2007; Bounhar, et al. 2006). Apoptosis induced in yeast by the Parkinson associated human α-synuclein protein has been utilized to identify yeast anti-apoptotic proteins Hsp70 and glutathione (Flower, et al. 2005). Many other anti-apoptotic proteins for yeast have been characterized (Osborn and Miller 2007).

1.8 Apoptosis, Yeast, and Human Disease

The use of yeast as a model for human disease has become widespread. Apoptosis can be a key mediator of human disease, and there are several models that utilize yeast apoptosis to further characterize the initiation and progression of cell death in these diseases (Mager and Winderickx 2005).

1.8.1 Parkinson's Disease and α-synuclein

Parkinson's disease is a common neurodegenerative disorder that is characterized by the loss of dopamine producing neurons (Dawson and Dawson 2003). The key morphological feature of these neurons is inclusion bodies, whose main component is α-synuclein (Spillantini, et al. 1998). An intrinsically unfolded protein, α-synuclein, has been shown to induce apoptosis in cultured neurons (Xu, et al. 2002). It was also shown that the toxicity leading to apoptosis affects dopamine production. The mechanism of action of this protein has not be fully elucidated though it is known that it forms fibres of various size, binds to lipids, inhibits the isoenzymes of phospholipase D, induces production of ROS, and leads to proteosomal inhibition (Lashuel, et al. 2002; Narayanan and Scarlata 2001; Jenco, et al. 1998; Flower, et al. 2005; Lindersson, et al. 2004). Investigation into the role of α-synuclein in Parkinson's disease has lead to the creation of yeast as models (Outeiro and Lindquist 2003). In *S. cerevisiae* α-synuclein has been shown to induce apoptosis and to increase the sensitivity to hydrogen peroxide mediated apoptosis (Flower, et al. 2005). Several factors exhibit an anti-apoptotic effect when co-expressed with α-synuclein in yeast including heat shock proteins such as Hsp70, and free radical scavengers like vitamin E and glutathione (Xu, et al. 2002).

1.8.2 Cardiac Disease

Apoptosis is a known cause of cardiac disease, especially under conditions of ischemia and reperfusion (Morissette and Rosenzweig 2005). Yeast has been

used to investigate the role of known proteins involved in cardiac disease (Nakajima, et al. 2007) or to develop interaction profiles through yeast two hybrids (Han, et al. 2006; Sun, et al. 2006). As well, knowledge gained about yeast genes has been applied to human genes involved in cardiac diseases to generate a better understanding of disease progression (Valianpour, et al. 2005).

1.8.3 Cancer and Apoptosis

The role of both splicing and apoptosis in the multifaceted disease of cancer has been extensively explored in other works (Pajares, et al. 2007; Fesik 2005; Stiewe 2007). In fact, the ability to evade apoptosis is recognized as a hallmark of the disease (Hanahan and Weinberg 2000). Cancer cells multiply without undergoing apoptosis when a genetic damage has occurred, due to broken repair mechanisms, or by bypassing the repair mechanism system. This leads to successive increases in mutations following each division as well as allowing uncontrollable cell growth. Yeast has been used to characterize the function of proteins whose regulation was known to be altered in cancerous cells (Benedetti, et al. 1998). To elucidate their function, human genes involved in cancer, such as the MYST genes, have been compared with their yeast homologues (Lafon, et al. 2007). Yeast is also being utilized as a tool to investigate and identify the molecular targets of novel anti-cancer drugs (Menacho-Márquez and Murguía 2007).

1.9 An Introduction to my Studies

The ability to evade apoptosis is an acquired characteristic that is associated with many normal and pathophysiological processes. These include oncogenesis and resistance to ischemia/reperfusion injury induced by pre-conditioning (Vermeulen, et al. 2005; Wang, et al. 2007). The unicellular budding yeast has been widely observed to undergo an apoptotic cell death which displays numerous similarities to that of metazoan cells (Madeo, et al. 2002). To further understand the molecular basis of anti-apoptosis we sought to identify novel anti-apoptotic sequences by screening mammalian cDNA expression libraries in yeast which conditionally express the mouse pro-apoptotic Bax (Yang, et al. 2006; Khoury, et al. 2007). My work consists of characterizing one of these Bax suppressors, originally termed Bh41, later identified as TMEM 85v2. TMEM 85v2 represents an alternatively spliced transcript from a novel human gene that currently has no known functions. In our hands, TMEM 85v2 serves to suppress the effects of Bax in yeast; therefore we sought to characterize this gene as a potential anti-apoptotic sequence. Using yeast-based viability and growth assays, I have confirmed that TMEM 85v2 overexpression reduces the apoptotic effects of hydrogen peroxide.

To characterize the gene corresponding to our isolated cDNA, TMEM 85, we compared its sequence to the GenBank database, revealing fifteen cDNA's that contain both identical and divergent sequences. This suggested that alternative splicing may be involved in the production of different TMEM 85 transcripts. Comparison of all the cDNA sequences with the human genomic sequence allowed us to determine the intron/exon composition of the human Bh41 gene.

RT-PCR analysis was performed using the RNA isolated from a panel of mice and human tissues and cell lines to show that all transcripts are expressed. A detailed analysis of the structure of this gene will be presented.

A decrease in anti-apoptotic gene expression is known to often lead to an increase in sensitivity to apoptotic stimuli (Schwerk and Schulze-Osthoff 2005). Yeast expresses an orthologue of the human TMEM 85 gene (YGL213c). Exploiting this knowledge, a yeast deletion mutant (YGL231cΔ) and yeast overexpressing this gene (using plasmid p426GAL1-YGL231c) were assessed through the viability and growth assays. We also used a yeast strain that expresses chromosomally TAP-tagged YGL231c to investigate the regulation of the gene.

YGL231c localizes to the ER (Huh, et al. 2003) and the deletion mutant is known to expresses a synthetically sick phenotype in the presence of α-synuclein (Willingham, et al. 2003), an inducer of ER stress. To investigate the possible role YGL231c, and by extension TMEM 85, might have in protecting against ER stress, over expression of human α-synuclein in both the wild type and deletion mutant was assessed.

Chapter 2: Experimental Procedures

2.1 Plasmids

The TMEM 85v2 clone was previously isolated by screening a human heart cDNA library regulated by a *GAL1* promoter in yeast cells expressing Bax (Yang, et al. 2006). The p426*GAL1*-YGL231c expressing plasmid was obtained from Open Biosystems (http://www.openbiosystems.com).

A p426 plasmid (*URA3* selectable marker) containing α-synuclein was obtained. To perform co-transformation it was necessary to place the α-synuclein in a plasmid with a different selectable marker (p425 plasmid with *LEU2* selectable marker). The p426- α-synuclein vector was transformed into *E. coli* using electroporation and selective growth on LB-Amp$^+$ plates. A single colony was grown and the plasmid isolated. The α-synuclein fragment was isolated using the restriction enzymes *Spe1/Xho1*. The fragment was cut from an agarose gel and purified using the QIAGEN's QIAquick Gel Extraction Kit. The isolated fragment was subcloned into a p425*GAL1* vector to generate a p425GAL1- α-synuclein plasmid. The plasmid empty p425*GAL1* was used as a control.

2.2 Yeast Strains, Media, Growth, and Transformation

The *S. cerevisiae* BY4741strain (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was used as the wild type. The strain was obtained from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). A *S. cerevisiae* knock out strain derived from BY4741 (*MAT***a**YGL231cΔ) and endogenously

promoted TAP-tagged yeast strains (YGL231c and YBL075c) were purchased from Open Biosystems. Yeast was grown in synthetic minimal media containing yeast nitrogen base (YNB), 2% glucose and supplemented with the required amino acids or bases. When expression of the *GAL1* promoter was required, the glucose was substituted with 2% galactose and 2% raffinose. Yeast was transformed with the appropriate plasmid using the lithium acetate method (Gietz, et al. 1992). The appropriate transformants were selected and maintained by omitting necessary amino acids or bases from the growth media which the vectors conferred auxotrophy (leucine for p425 vectors, uracil for p426 vectors). YEPD media consists of 2% bactopeptone, 1% yeast extract, and 2% glucose. Solid media contained 2% agar.

2.3 Viability Assay

A modified colony forming unit (CFU) assay was used to assess cell viability (Yang, et al. 2006). The transformed yeast was grown overnight in minimal media containing glucose. Cells were diluted in fresh minimal media containing either glucose or galactose and grown for two hours to ensure cells were in exponential phase. The yeast was grown to a cell density of $1x10^6$ cells /ml and subjected to one of the following known inducers of cell stress. For all, the number of colonies formed on nutrient agar plates was compared between yeast expressing and not expressing the TMEM 85v2 plasmid during exposure, and recorded as a percentage of colonies formed in untreated cells.

Reactive oxygen species, produced by exogenous hydrogen peroxide, are a known mediator of yeast apoptosis (Madeo, et al. 2002). The yeast was treated with the indicated amount of hydrogen peroxide for four hours. After treatment, the cells were plated on YEDP and grown for two days at 30°C. Irreparable DNA damage due to UV induces apoptosis in yeast (Del Carratore, et al. 2002). Yeast cells were plated on YEPD, then treated with 350x100μJ/cm2 UV and grown for two days at 30°C. Defects in ER stress, induced by defects in N-glycosylation, result in apoptosis (Hauptmann, et al. 2006). DTT induces ER stress by preventing the formation of disulphide bonds. Yeast was treated with 35mM of DTT for four hours. After treatment, the cells were plated on YEPD and grown for two days at 30°C. Tunicamycin induces ER stress by inhibiting protein glycosylation. Yeast was treated with 0.01μg/ml of tunicamycin for four hours. After treatment, the cells were plated on YEPD and grown for two days at 30°C. α-synuclein is known to induce apoptosis by a variety of mechanisms. Yeast harbouring p425α-synculein alone and other plasmids were grown for four hours in galactose media in order to induce α-synuclein expression. After treatment, the cells were plated on YEDP and grown for two days at 30°C.

2.4 Hydrogen Peroxide Halo Assay

Yeast harbouring the p426*GAL1*- TMEM 85v2 plasmid were grown overnight in minimal media containing glucose. Cells were outgrown the following day for two hours in minimal media containing either glucose or galactose. A final cellular concentration of $1x10^6$ cells /ml was reached and 200μl of these cultures

was mixed with a pre-warned 5ml of 0.5% soft agar containing minimal media with either glucose or galactose. The soft agar-yeast mixture was plated on solid minimal media containing glucose or galactose and let dry four hours. A sterile filter disk with 2µl of 30% hydrogen peroxide was then added to the dried plate. Yeast was grown for three to four days at 30°C. Results are indicative of at least three independent experiments.

2.5 Spot assay

Yeast expressing the p426 galactose inducible plasmid containing TMEM 85v2 were grown overnight in minimal media containing glucose. Cells were outgrown the following day for two hours in minimal media containing either glucose or galactose to a final cellular concentration of $1x10^6$cells/ml. Aliquots of 10µl of a one in seven serial dilutions in water were spotted on glucose or galactose minimal media agar plates (Yang, et al. 2006). The yeast was grown two to three days at 30°C. Results are indicative of at least three independent experiments.

2.6 RT-PCR

RNAzol was used to extract RNA from cultured cells and tissue samples as previously described (Yang, et al. 2005). Using the ThermoScript RT-PCR system (Invitrogen), RNA was reverse transcribed and amplified by PCR. The *TMEM 85* alternative transcripts were amplified using equal aliquots of cDNA under the following conditions: 94°C for 30s, 57° C for 30s, 72°C for 40s, for a total of 35 cycles. The forward primers were 5'-GAAGCATCGAGGCTATAGGAC-3' used for TMEM 85v1, v2, and v3, and 5'-

CTGGAAAACCTATAGGGAA-3' for TMEM 85v4. The reverse primers were 5'-AATGAAGGGTAACCAATCCGA-3' used for TMEM 85v1 and v3, and 5'-CAGTCCTCCACCACTGAACTC-3' for TMEM 85v2, and 5'-GCAGGCAGATCGCTTGAGCCC-3' for TMEM 85v4 . β-*ACTIN* mRNA was amplified using the forward: 5′-GTGGGCCGCCCTAGGCACCAG-3′ and reverse: 5′-CTCTTTGATGTCACGCACGATTTC-3′ primers as previously described (Yang, et al. 2005). An aliquot of each PCR reaction was separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, visualized and photographed under UV illumination.

2.7 Protein Extraction and Western Blot Analysis

Yeast was grown overnight in minimal media containing glucose. Cells were diluted the following day and grown for one hour in minimal media containing either glucose or galactose. The yeast was grown to a cell density of $1x10^6$cells/ml and grown in glucose or galactose for five hours. Cells were washed in sterile water and then treated with 0.2M NaOH for five minutes. Protein was extracted from the treated yeast by boiling (95°C) for five minutes in SDS-PAGE yeast loading buffer (0.06M Tris-HCL ph6.8, 5% glycerol, 2% SDS, 4% β-mercaptoethanol, and 0.0025% bromophenol blue) (Kushnirov 2000). Proteins were separated using SDS-PAGE (12% acrylamide; 37.5 acrylamide: 1bisacrylamide) and transferred to a nitrocellulose membrane electrically overnight. The membrane was rinsed for five minutes with cold TBS (Tris 0.01 M, ph 7.4, 0.15 M NaCl), then blocked for one hour with a milk solution (5% fat free powdered milk, 1% Tween, in TBS). The membrane was challenged with

rabbit anti-TAP antibody (1/1000 dilution) at 4°C in fresh milk solution for two hours. The membrane was rinsed three times for five minutes each time with cold TBS-T (1% Tween in TBS). After rinsing, the blot challenged for one hour in HRP conjugated goat anti-rabbit IgG antibody (1/10 000 dilution) at 4°C in fresh milk solution. This was followed by six, five minute washes in cold TBS-T. The blot was exposed to ECL Plus (Amersham Bioscience) for five minutes activation of the signal on the secondary antibody. Detection was performed by exposure to X-ray film (Kodak X-Omat). Results are indicative of at least three independent experiments.

Chapter 3: Genomic and Functional Characterization of TMEM 85

3.1 TMEM 85v2 acts as an inhibitor of hydrogen peroxide induced stress

Our lab has been interested in identifying novel mammalian anti-apoptotic proteins by screening of cDNA libraries in yeast cells expressing mammalian Bax (Yang, et al. 2006; Khoury, et al. 2007). To carry out the screens yeast were transformed with the pGILDA vector containing mouse Bax (Xu, et al. 2000). This placed expression of the Bax cDNA under the control of a galactose inducible promoter. Bax is known to induce cell death in yeast in a manner highly similar to mammalian cellular apoptosis (Ligr, et al. 1998). A human cardiac cDNA library cloned into the galactose-inducible pYES-DEST52 vector. The vector library was co-transformed with the Bax vector in yeast and grown on galactose containing media. Colonies that were protected against Bax induced apoptosis were isolated and the resultant cDNA's were sequenced. Sixty two anti-apoptotic clones were thus identified, many of which are known anti-apoptotic proteins such as Hsp72. Our lab has previously characterized three of these clones, namely SMS1α, Vps24β, and TSC22 as representing novel anti-apoptotic sequences (Yang, et al. 2006; Khoury, et al. 2007; unpublished data). Here, we aim to characterize another Bax suppresser that corresponds to a poorly characterized novel 149aa protein that has been identified as TMEM 85. Our

TMEM 85 likely represents a novel splice variant that we have named TMEM 85v2 (see below)

Although yeast undergoes apoptosis like death in response to Bax, yeast apoptosis is also known to be induced by a variety of other stimuli. Hydrogen peroxide is possibly the most used and effective method of inducing apoptosis in yeast due to the central role of ROS (Madeo, et al. 2004). The ability of TMEM 85v2 to prevent hydrogen peroxide mediated cell death was examined to independently verify the protective nature of our clone. Yeast cultures containing either an empty vector, a plasmid expressing a known anti-apoptotic sequence (BS. 32) also identified in the screen (Khoury, et al. 2007), or a p426-TMEM 85v2 plasmid were treated with 1.5mM hydrogen peroxide for four hours. Based on previous work in our lab, this concentration of hydrogen peroxide is known to induce conditions of stress leading to apoptosis and not necrosis (Yang, et al. 2006; Khoury, et al. 2007). Viability was determined using a colony forming unit assay (CFU assay). This methodology is recognized as serving as a basic assay to monitor cell death (Fröhlich, et al. 2007). Only viable cells give rise to a colony, indicating a yeast cell that has successfully survived the stress conditions. In the absence of hydrogen peroxide, viability for all three strains was close to 100% (Fig. 1A). In the presence of hydrogen peroxide, the viability of control cells harbouring empty vector was decreased to 22.2% ± 5% (Fig. 1A). In contrast viability was increased to respective 58% ± 3.5% and 60.2% ± 0.4%, in BS.32 and TMEM 85 (Fig. 1A). This indicates that TMEM 85 was able to protect the yeast cells from death.

A halo assay was performed as a separate and independent technique in order to evaluate the ability of TMEM 85v2 to protect yeast cells from hydrogen peroxide. Yeast will not grow in a region which contains too high a concentration of hydrogen peroxide. This area of no growth is termed the halo which surrounds a disc containing the stress factor. A smaller halo is indicative of a yeast strain being more able to withstand the inhibitory effects of hydrogen peroxide. The halo assay has previously been used in this manner to demonstrate the killing effect of hydrogen peroxide (Flower, et al. 2005). Equal numbers of wild type yeast containing either empty vector or the galactose inducible human TMEM 85v2 plasmid were plated on glucose and galactose minimal media. The assay confirmed the results seen in the CFU hydrogen peroxide assay (Fig.1E); the halo surrounding the empty vector is much larger than either of the halo around the wild type yeast expressing human TMEM 85v2.

We also wanted to determine if TMEM 85v2 protects cells from other forms of stress that are known to induce apoptosis. We have previously found that there are differential abilities of our anti-apoptotic clones to prevent cell death with different stressors (Yang et al. 2007; Khoury et al. 2007). UV light is known to induce apoptosis in yeast (Del Carratore, et al. 2002). To examine if TMEM 85 mediated its effects through either DNA damage repair or prevention of UV induced DNA damage, each of the yeast strains were exposed to UV light. No significant protective effect was seen in either BS.32 or TMEM 85v2 expressing yeast (Fig. 1B). The yeast homologue of human TMEM 85 is known to be localized to the ER (Huh, et al. 2003). Therefore, it was hypothesised that TMEM

85v2 may mediate its anti-apoptotic effects through the ER. Both DTT and tunicamycin are known to induce ER stress. Again, no significant protective effect was seen in either the BS. 32 or the TMEM 85v2 expressing yeast (Fig. 1C and D).

3.2 Genomic Organization and Alternative Splicing of Human *TMEM 85*

Comparison of cDNA with the sequences available in the GenBank databases revealed fifteen corresponding cDNA's. Eleven of these showed identity to each other (Accession Number: CR599971, CR595333, AK075227, CR607532, CR597763, CR619572, AF151018, NM_016454, CR597809, CR620239, AY336092); however our clone contained a unique 3' end as well as some novel sequences. This suggested that alternative splicing may lead to the production of different TMEM 85 transcripts. One of the cDNA's was found to contain a sequencing error leading to a frame shift (BC016348). Of the remaining three cDNA's, one corresponds to our clone (BC002583) while the other two presented as unique sequences with some regions of identity.

To determine the origins of the different cDNA's and the intron/exon composition of the human TMEM 85 gene, we used BLAST to compare the cDNA sequences with the human genomic sequence, reference assembly. We found that the TMEM 85 gene is spread over 5.1 Kb on human chromosome XV (AC079203). It consists of five different exons that are alternatively spliced to produce at least four different mRNA's and proteins (TMEM 85v1, TMEM 85v2, TMEM 85v3,

and TMEM 85v4). A nomenclature was adopted to differentiate between the different TMEM 85 transcripts and their corresponding proteins (Fig. 2A). While exons 1, 2, 3B and 5 all follow the GT/AG splicing rule-they are spliced by the U2 spliceosome, exon 3A appears to follow the splicing determinants of the U12 spliceosome, AT/variable 3' splice site (Table 1).

Eleven cDNA sequences correspond to the putative protein, TMEM 85v1, indicating it is likely the most commonly expressed isoform. It consists of exons 1, 2, 3B, 4, and 5B, and its protein is 183 amino acids in length. Using several data mining tools available through GenBank and ExPASy (http://expasy.org), TMEM85 v1 was determined to contain two transmembrane domains and the DUF 1077 domain that represents a conserved domain of unknown function. The transmembrane domains are predicted to have orientation with the N- and C- term facing inside the membrane (Fig. 2 B). Although the available cDNA's contain no confirmed polyA tail, there is a sequence that does contain two putative polyA signals (AAUAAA/ACUAAA). This putative TMEM 85 protein also contains a complete DUF1077 domain but no other recognized functional domains.

Our TMEM 85 sequence isolated in the Bax viability screen corresponds to a mRNA produced from the TMEM 85 gene that we have called TMEM 85v2 (Fig. 2A,B). The TMEM 85v2 protein contains one transmembrane domain. The transcript is composed of exons 1, 2, 3B, and 5B, and produces a protein 143 amino acids in length. It contains most of the DUF 1077 domain, lacking only a small portion at the C-term, and no other recognizable domains. Resulting from

the lack of exon 4, there is a frame shift in the coding region of v2, giving rise to a unique C-term sequence.

The TMEM 85v3 putative transcript contains exons 1 and 5B only, producing a protein 87 amino acids in length (Fig, 2A, B). The protein contains no transmembrane domain. Only one GenBank entry corresponds to this putative protein (CR609616). This is the only putative protein to completely lack the DUF1077 domain, as well as any other known functional domain.

The cDNA sequence of the putative TMEM 85v4 protein, the shortest of the proteins produced consists of exon 3A and all of exon 5 (Fig. 2A, B). Only one GenBank entry corresponds to this sequence (AK091678), but no protein sequence based on this cDNA had yet been confirmed. Using ORF Finder, the most likely open reading frame (ORF) for this protein was deduced. The ORF results in a 66 amino acid protein that contains a short region of the DUF 1077 domain seen in v1 and v2 (Fig. 2B).

To verify that each of the putative alternatively spliced transcripts are expressed in human cells, semi-quantitative RT-PCR was performed on a panel of human tissues and cells lines (Fig. 2C). Primers which allowed identification of the unique identification of transcripts were designed and are shown in figure 2A. Primers were designed for exons 1, 3A, 5, and two for 5B. Two primers were designed for 5B due to the frame shift in v2 which provides a unique C-term. TMEM 85 v1, v2, and v3 were all identifiable in human heart. TMEM 85v1 and v3 were found in brain tissue, and v1 was found alone in HEK293 cells. TMEM

85v4 was not identified in any of the samples tested. These results confirm that TMEM 85v1, v2, and v3 are expressed transcripts. The uncommon and weak mechanism of splicing of TMEM 85v4 indicates that its detection may be extremely difficult due to low cellular expression except under very specific conditions. It is more likely that TMEM 85v4 is not expressed in the human tissues tested in this study, although TMEM 85v4 may not be a real transcript.

3.3 Genomic Organization and Alternative Splicing of Mouse *TMEM 85*

The human TMEM 85 has homologues in many other species. Mouse tissue was used to further investigate putative tissue distribution of the different transcripts, as we did not have easy access to human tissues. Examination of the GenBank database revealed six mouse cDNA sequences corresponding to the human TMEM 85 gene. All of these sequences corresponded to human TMEM 85v1, no cDNA sequences had been entered for TMEM 85v2, v3, or v4. To ensure a true homologue existed to human TMEM 85, the genomic organization of mouse TMEM 85 was elucidated. Using the cDNA sequences and BLAST, the genomic location, intron/exon borders and sizes for exons 1, 2, 3A, 4, and 5 were determined (Fig. 3A; Table 2). Mouse TMEM 85 exists as 5Kb gene located on chromosome II. Using the BLAST search engine as well as the EST database, the corresponding mouse genomic region to human exon 3A was determined. While no mouse cDNA or protein is currently imputed in GenBank containing exon 3A, identity exists between region of the human TMEM 85 gene that contains exon

3A and the corresponding region in the mouse TMEM 85 gene. Based upon this in silco analysis of the possible mouse genomic organization of TMEM 85, the four transcripts that correspond to human TMEM 85 are also present in the mouse TMEM 85 gene. Mouse TMEM 85 theoretically produces four proteins of equal size and with the same domains and features.

To determine tissue localization, semi-quantitative RT-PCR was performed on a panel of mouse tissues (Fig. 3B). The same primers were used for the RT-PCR, due to the identity between the human and mouse exons. Four bands, corresponding to the predicted size of each of the transcripts, were only found in testis. Both transcripts corresponding to mouse TMEM 85v1 and v4 were found in all tissues tested. Of note there appeared to be upregulation of both transcripts in C2C12, a mouse skeletal muscle cell line, when the cells were treated with TNF-α, a known inducer of cellular stress. However, none of the transcripts have yet been confirmed by sequencing. Therefore, no definite conclusions about tissue distribution can be made.

3.4 Yeast TMEM 85 protects against hydrogen peroxide induced stress

A BLAST search of the human TMEM 85 protein against the yeast proteome, revealed a likely homologue, YGL231c. YGL231c is located on chromosome VII and as almost all yeast genes contains no introns. There is 26% identity between the TMEM v1 and YGL231c proteins but only 20% identity with the TMEM v2 protein (Fig. 4A). As well, v1 is almost identical in length to YGl231c (183 vs.

190 residues) and shares homology through out its sequence. Therefore, it is likely that YGL231c is actually a homologue to human TMEM 85v1. Exploiting this knowledge, a yeast deletion mutant (YGL231cΔ) and a plasmid overexpressing the yeast gene (p426*GAL1*-YGL231c) were used to determine if the yeast gene is also anti-apoptotic. A CFU viability assay using 1.5mM hydrogen peroxide, 35mM DTT, or 0.01μg/ tunicamycin was performed to determine if the deletion strain was more sensitive, as a decrease in anti-apoptotic gene expression often leads to increased sensitivity to apoptotic stress (Spee et al.2006). The viability assay indicated that the mutant lacking this gene does not show increased apoptosis when compared to wild type yeast cells (Fig. 4B). The halo assay was used to assess the protective effect of overexpression of YGL231c. Using the halo assay, yeast cells harbouring p426*GAL1*-YGL231c show a galactose dependent increase in growth around filter discs containing hydrogen peroxide (Fig. 4C). Overexpression of YGL231c does therefore protect yeast from hydrogen peroxide induced apoptosis. We also found that mutant cells lacking YGL231c are protected from hydrogen peroxide by overexpression of TMEM 85v2 (Fig. 4C). This indicates that TMEM 84 does not require endogenous YGL231c for its anti-apoptotic effects.

3.5 TAP-Tagged YGL231c is not Upregulated Under Stress Conditions

To investigate the possibility of induction of the TMEM 85 gene during conditions of stress, a yeast strain containing a TAP-tagged YGL231c gene was

examined. Yeast was exposed to increasing concentrations of hydrogen peroxide, followed by examination of the expression of YGL213c-TAP protein. Under low levels of apoptotic stress, anti-apoptotic genes and pathways are often unregulated in an attempt to protect the cell. A western blot was performed on the yeast proteins extracted after hydrogen peroxide treatment (Fig. 5A). TAP-tagged YGL231c was detected at similar levels in all samples examined indicating that the gene is unlikely to be regulated by pro-apoptotic stimulus (Fig. 5A). TAP-tagged YBL075c (Hsp72) was used as a control for the specificity of the TAP antibody (Fig. 5A). A CFU viability assay was also performed on the yeast to confirm that the concentration of hydrogen peroxide used leads to increasing cells death (Fig. 5B).

3.6 TMEM 85v2 does not Protect Against α-synuclein Mediated Apoptosis

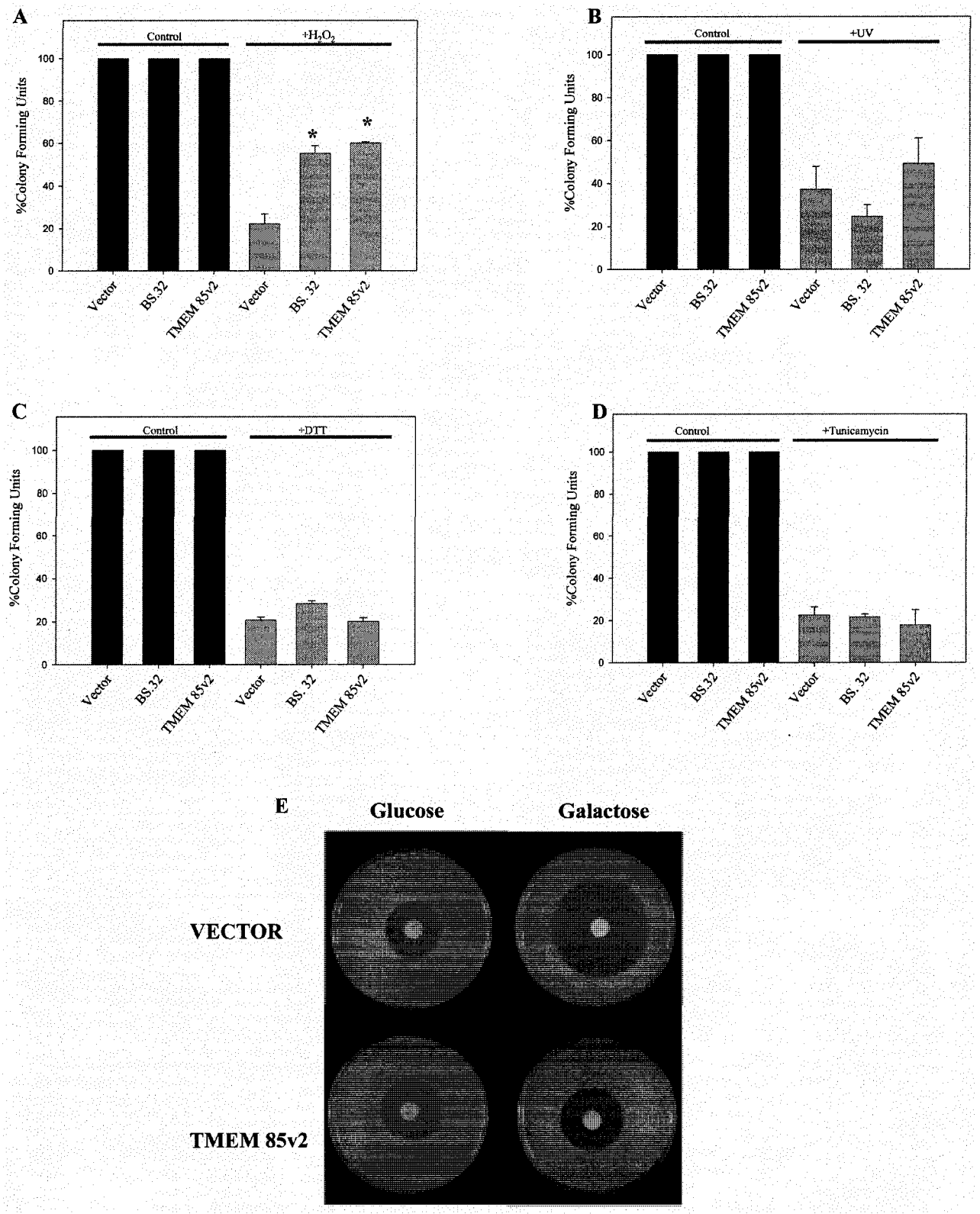
Earlier studies had indentified that yeast strains lacking their endogenous homologue of human TMEM 85, YGL231c, were synthetically lethal under certain conditions in the presence of α-synuclein (Willingham, et al. 2003). One of the effects of α-synuclein is to induce ER stress through the production of unfolded proteins. YGL231c is known to localize to the ER (Huh, et al. 2003). It therefore seemed possible that TMEM 85 mediated its anti-apoptotic effect through reduction of ER stress. Wild type yeasts transformed with a plasmid expressing α-synuclein alone show a significant decrease in viability (Fig 6A).

Overexpression of TMEM 85v2 is not able to prevent or significantly reduce the amount of yeast death due to α-synuclein. A spot assay of the strains further confirms that no difference exists between the growth of yeast expressing α-synuclein in the presence or absence of TMEM 85v2 (Fig. 6B).

As the YGL231c deletion mutant was previously shown to be more sensitive to α-synuclein induced stress, the deletion strain was also transformed with α-synuclein. While α-synuclein did result in yeast apoptosis, overexpression of TMEM 85v2 was also unable to increase cell survival in cells lacking YGL231c (Fig. 6C).

Figure 1. Human TMEM 85v2 suppresses the loss of viability and the growth inhibitory effects of hydrogen peroxide.

Yeast cells were transformed with empty vector, positive control (BS. 32), or TMEM 85v2 expressing vector and treated with 1.5mM H_2O_2 (A), 350X100μJ/cm2 UV(B), 0.01μg/ml tunicamycin(C), or 35mM DTT(D). Viability was compared between yeast expressing and not expressing the plasmids during exposure to stress, and recorded as a percentage. Data are ± the standard error and represent a minimum of three independent experiments. "*" indicates that the viability of BS. 32 and TMEM 85v2 expressing cells is significantly different than cells harbouring empty vector, by student t-test ($p<0.001$) (E) Halo assays were performed with yeast transformed with empty vector or TMEM 85v2. The transformants were grown on glucose or galactose containing agar plates. The central disc contained 2μl of 30% hydrogen peroxide. Increased sensitivity to the growth inhibitory effects of hydrogen peroxide presents as a larger zone of no growth.


A
Control
+H2O2
%Colony Forming Units
100
80
60
40
20
0
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Vector
BS.32
TMEM 85v2
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BS. 32
TMEM 85v2
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+UV
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TMEM 85v2
Vector
BS. 32
TMEM 85v2
C
Control
+DTT
%Colony Forming Units
Vector
BS.32
TMEM 85v2
Vector
BS. 32
TMEM 85v2
D
Control
+Tunicamycin
%Colony Forming Units
Vector
BS.32
TMEM 85v2
Vector
BS. 32
TMEM 85v2
E
Glucose
Galactose
VECTOR
TMEM 85v2

Figure 2. Schematic Representation of the Human TMEM 85 Gene and Expression Analysis

(A) Genomic organization of the human TMEM 85 gene. The TMEM 85 gene consists of five exons located on chromosome 15q14. Exons are shown as coloured boxes that are numbered. Introns that separate the exons are shown as lines. Exon three contains two regions, A and B, which are never expressed concurrently and contain a 132bp region of overlap (green). Exon five also contains two regions. From this 5.1Kb gene, four putative mRNA transcripts are produced, v1-v4. Arrows indicate the position of RT-PCR primers (a-d). Regions of identity are indicated by colour, transmembrane domains are indicated in the protein (pink). (B) Schematic representation of the four putative proteins produced by the TMEM 85 gene. TMEM 85v1 and v2 contain transmembrane domains. TMEM 85v3 and v4 do not harbour transmembrane domains. Areas of identity between different proteins are indicated by the use of the same colour. (C) RT-PCR analysis of the TMEM 85 transcripts in human tissues and cell lines. Total RNA was isolated from human tissues, reverse transcribed, and the different isoforms were amplified using the oligos shown in figure 3A. Oligo's a through d were used to amplify the differently sized TMEM 85v1, v2, and v3. β-*ACTIN* was also amplified as a control. An aliquot of each PCR reaction was separated by agarose gel electrophoresis, stained with ethidium bromide and a composite of the resultant photographs are shown. The size in base pairs (bp) of each PCR product corresponding to the predicted size of the different TMEM 85 transcripts is shown on the right. Similar results were obtained in two separate experiments.

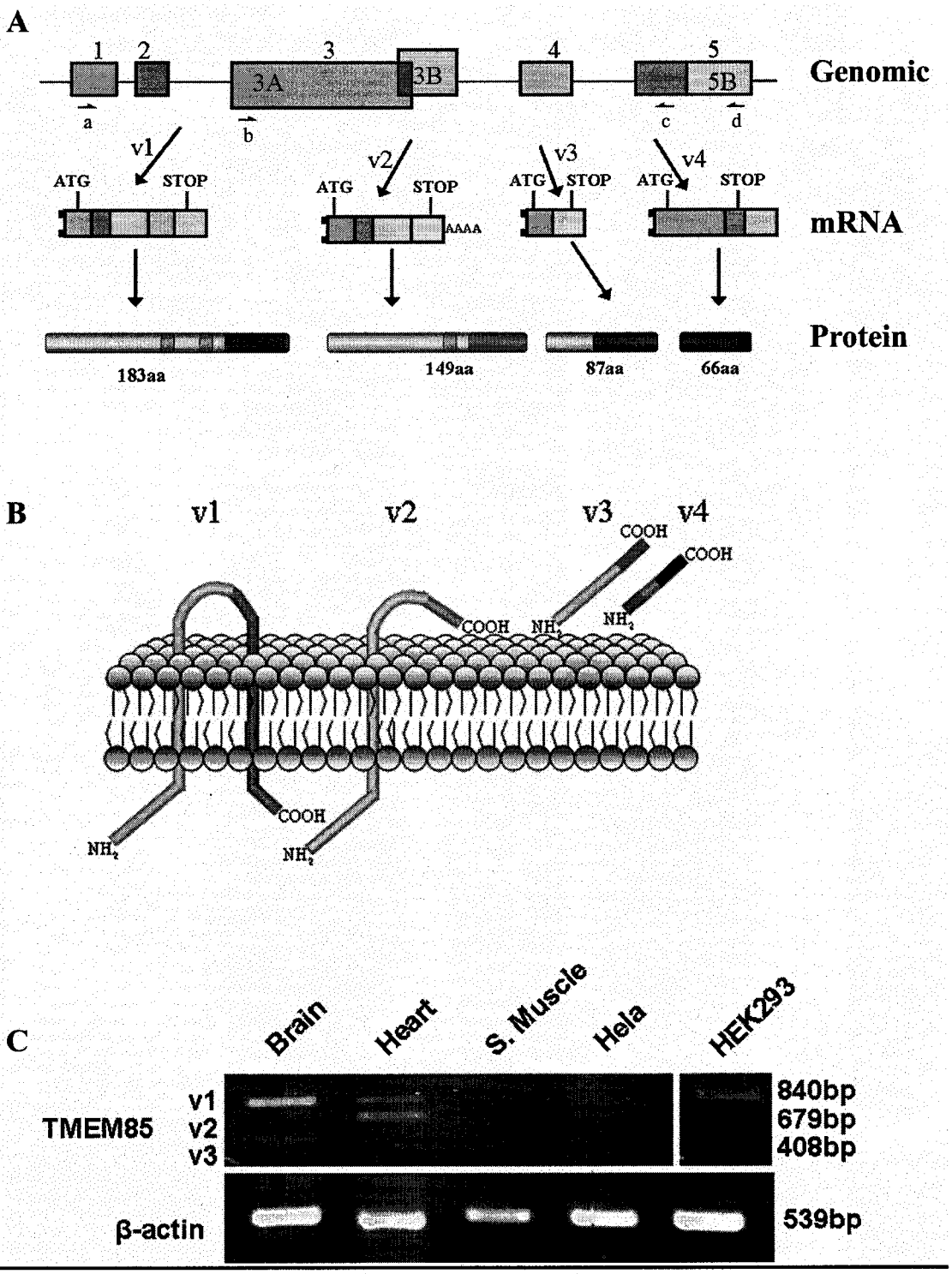

A
1
2
3
3A
3B
4
5
5B
Genomic
a
b
c
d
v1
v2
v3
v4
ATG
STOP
AAAA
mRNA
Protein
183aa
149aa
87aa
66aa
B
v1
v2
v3
v4
COOH
NH2
C
Brain
Heart
S. Muscle
Hela
HEK293
TMEM85
v1
v2
v3
840bp
679bp
408bp
β-actin
539bp

Table 1. Sequence of intron/exon junctions of the alternatively spliced human TMEM 85 gene.

The intron/exon borders were determined by comparing the cDNA sequences found through BLAST with the human genome sequence. Introns are shown as lower case, exons as upper. There is no intron size for exon 3A as it overlaps with exon3B. No intron size is shown for exon 5A, as it is always spliced with 5B.

Exon No.	Splice Acceptor Site (Intron/Exon border)	Exon (bp)	Splice Donor Site (Exon/Intron Border)	Intron(bp)
1	5'end-ACAAAGCGGA	169	GAGGCAGCAGgtgaggcacc	336
2	ggtttttttagGGGTCGAAGT	115	GGTGGAGAAGgtacagaggt	862
3A	ttcttgaaatGGATGCTTAG	1313	ACCCATTACAGgcacttatgg	
3B	tttgttttagCGCTGCTGGG	156	ATTTCAGCCAgtaagtattc	583
4	accgcaacagCTTTCAAGAT	161	GCCCCCTGAGgtaagcaaa	855
5	ccgggctcagGCACTCAGCC	713	AACAATGCATaaaaaaagtt	
5B	ctttctttcagAGAATGGAGT	404	CATAAAAAAA-3'end	

Figure 3. Schematic Representation of the Mouse TMEM 85 Gene and Expression Analysis

(A) Genomic organization of the mouse TMEM 85 gene. The TMEM 85 gene consists of five exons located on chromosome II. Exon three contains two regions, A and B, which are never expressed concurrently and contain a 132bp region of overlap (green). Exon five also contains two regions. From this 5Kb gene, four putative transcripts are produced, v1-v4. The TMEM 85 putative transcripts all contain part or the entire DUF1077 domain. Arrows indicate the position of RT-PCR primers. Regions of identity are indicated by colour, transmembrane domains are indicated in the protein (pink). (B) RT-PCR analysis of the TMEM 85 transcripts in a panel of mouse tissue. Total RNA was isolated from mouse tissues, reverse transcribed, and the different isoforms were amplified. The primers were identical to those used in human tissue. β-*ACTIN* was also amplified as a control. An aliquot of each PCR reaction was separated by agarose gel electrophoresis, stained with ethidium bromide and a composite of the resultant photographs are shown. The size in base pairs (bp) of each PCR product corresponding to the predicted size of the different TMEM 85 transcripts is shown on the right. Similar results were obtained in two separate experiments.

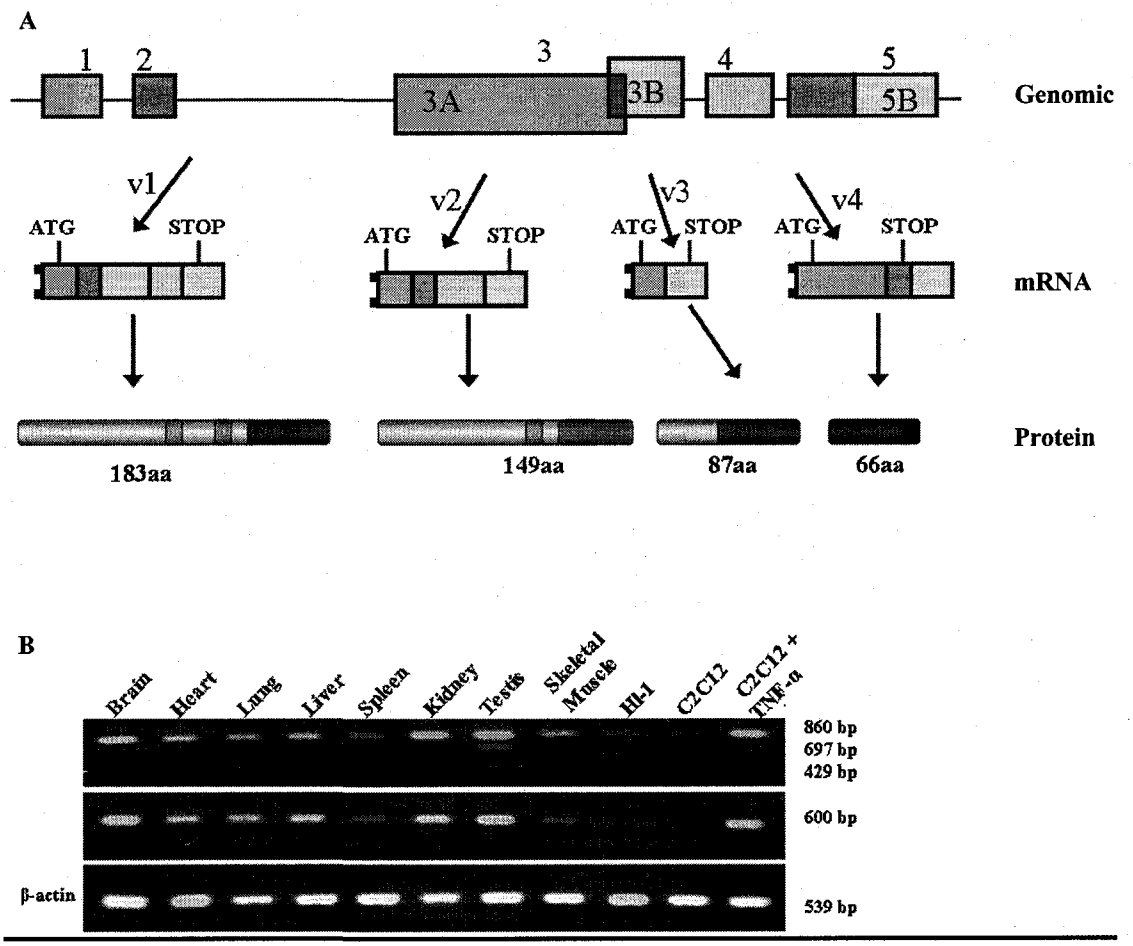

A
1
2
3
3A
3B
4
5
5B
Genomic
v1
v2
v3
v4
ATG
STOP
mRNA
183aa
149aa
87aa
66aa
Protein
B
Brain
Heart
Lung
Liver
Spleen
Kidney
Testis
Skeletal Muscle
HI-1
C2C12
C2C12 + TNF-α
860 bp
697 bp
429 bp
600 bp
β-actin
539 bp

Table 2. Sequence of intron/exon junctions of the alternatively spliced mouse TMEM 85 gene.

The intron/exon borders were determined by comparing the cDNA sequences found through BLAST, the human exon 3A sequence and the EST database with the mouse genome sequence. Introns are shown as lower case, exons as upper. There is no intron size for exon 3A as it overlaps with exon3B. No intron size is shown for exon 5A, as it is always spliced with 5B.

Exon No.	Splice Acceptor Site (Intron/Exon border)	Exon (bp)	Splice Donor Site (Exon/Intron Border)	Intron (bp)
1	5'end-ATGCGCCGGA	209	GAGGCAGCAG**gt**gagtctct	303
2	ggttctttt**ag**GGGCCGAAGT	115	GGTGGAGAAG**gt**acagaggt	1770
3A	agtcttttt**ag**GGTTTGGGTA	1456	ACCCATTCAAgcacttatgg	
3B	tttatttt**at**CGCTGCTGGG	154	ATTTCAGCCA**gt**aagtattc	200
4	atcccaacc**ag**CTTTCAAGAT	161	GCCCCCTGAG**gt**atggttaa	141
5	gaggtcag**ag**AACTTGGTGC	321	GCTTACTTCT**gt**gaccccca	
5B	tctttttttt**ag**AGAATGGAGT	336	GAGAACAATG-3'end	

Figure 4. Comparison of Human TMEM 85 and its Yeast Homologue YGL231c

(A) Comparison of the amino acid sequence of human TMEM 85v1 and TMEM 85v2 with yeast YGL231c. Regions of identity between all three sequences are shown in blue, between TMEM 85v2 and YGL231c are shown in red, between TMEM 85v2 and YGL231c are shown in green. Gaps (-) are introduced to maximize the alignment.(B) Deletion mutant yeast (YGL231cΔ) and wild type yeast were treated with 1.5mM H_2O_2, 35mM DTT, or 0.01μg/ml tunicamycin. Viability was compared between yeast exposed and not exposed to the stresses, and expressed as a percentage of cells that formed colonies. Data are ± the standard error and represent a minimum of three independent experiments. (C) Halo assays were performed with wild type or deletion mutant (YGL231cΔ) yeast transformed with empty vector or with vector expressing YGL231c or TMEM 85v2 under the control of a galactose inducible promoter. The central disc contained 2μl of 30% hydrogen peroxide. Increased sensitivity to the growth inhibitory effects of hydrogen peroxide presents as a larger zone of no growth.

A

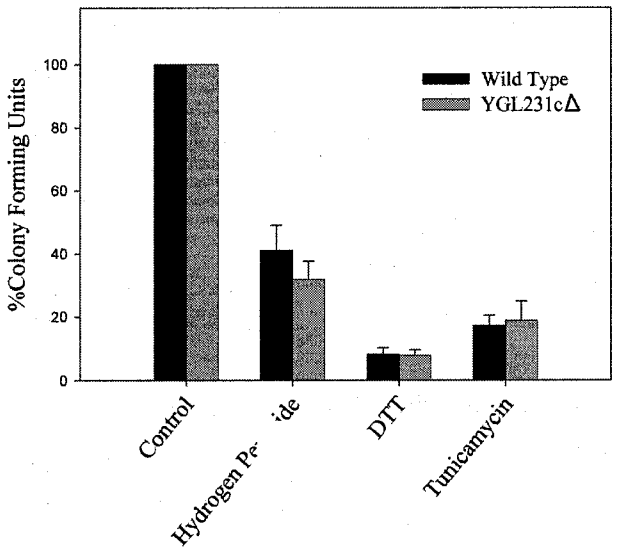
```
v1/v2  MTAQG-----GLVANRGRRFKWAIELSGPGGGSRGRSDRGSGQGDSLYPVGYLDKQVPDT 55
Yeast  MSEQEPYEWAKHLLDTKYIEKYNIQNSNTLPSPPG-FEGNSSKGNVTRKQQDATSQTTSL 59

v1/v2  SVQETDRILVEKRCWDIALGPLKQIPMNLFIMYMAGNTISIFPTMMVCMMAWRPIQALMA 115
Yeast  AQKNQITVLQVQKAWQIALQPAKSIPMNIFMSYMSGTSLQIIPIMTALMLLSGPIKAIFS 119

v2     ISA--KNGVQWW------RTAFVNMRKQRLVPMYLGLIYILL                    147
v1     ISATFKMLE----SSSQKFLQGLVYLIGNLMGLALAVYKCQSMGLLPTHASDWLAFIEPP 171
Yeast  TRSAFKPVLGNKATQSQVQTAMFMYIVFQGVLMYIGYRKLNSMGLIPNAKGDWL----PW 177

v1     ERMEFSGGGLLL--- 183
Yeast  ERIAHYNNGLQWFSD 190
```

B



C

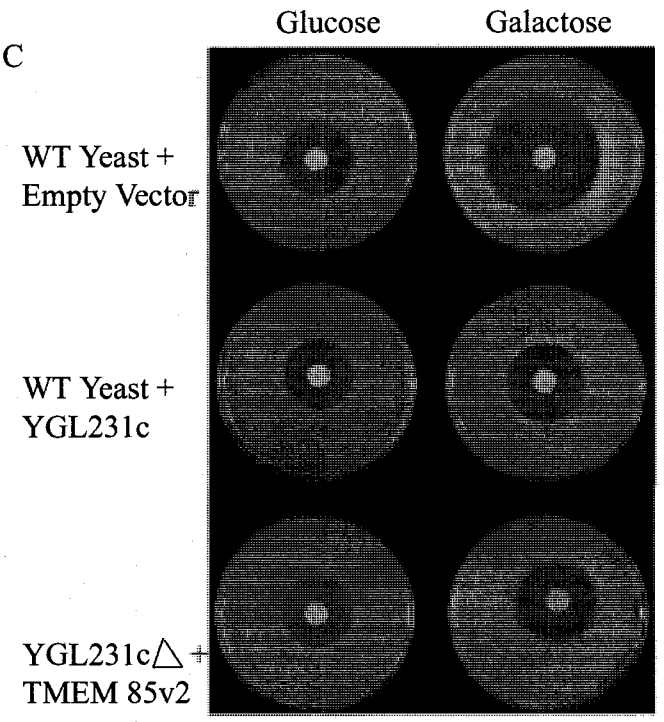


Figure 5. YGL231c is not upregulated during exposure to hydrogen peroxide. (A)Western blot analysis of extracts prepared from YGL231c TAP-tagged yeast treated with the indicated concentration of H_2O_2. Identical quantity of protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membrane, and challenged with rabbit anti-TAP. A single band corresponding to TAP-tagged YGL231c was detected. TAP-tagged YBL075c was used as a control. Molecular weight markers are shown on the left. Results are representative of at least three independent experiments (B) Viability assay of yeast cells used for western blot analysis. Yeast cells containing chromosomally TAP-tagged YGL231c treated with the indicated concentration of H_2O_2. Viability was determined for yeast exposed to H_2O_2, and presented as the percentage of cells that remained viable after treatment. Data are ± the standard error and represent a minimum of three independent experiments

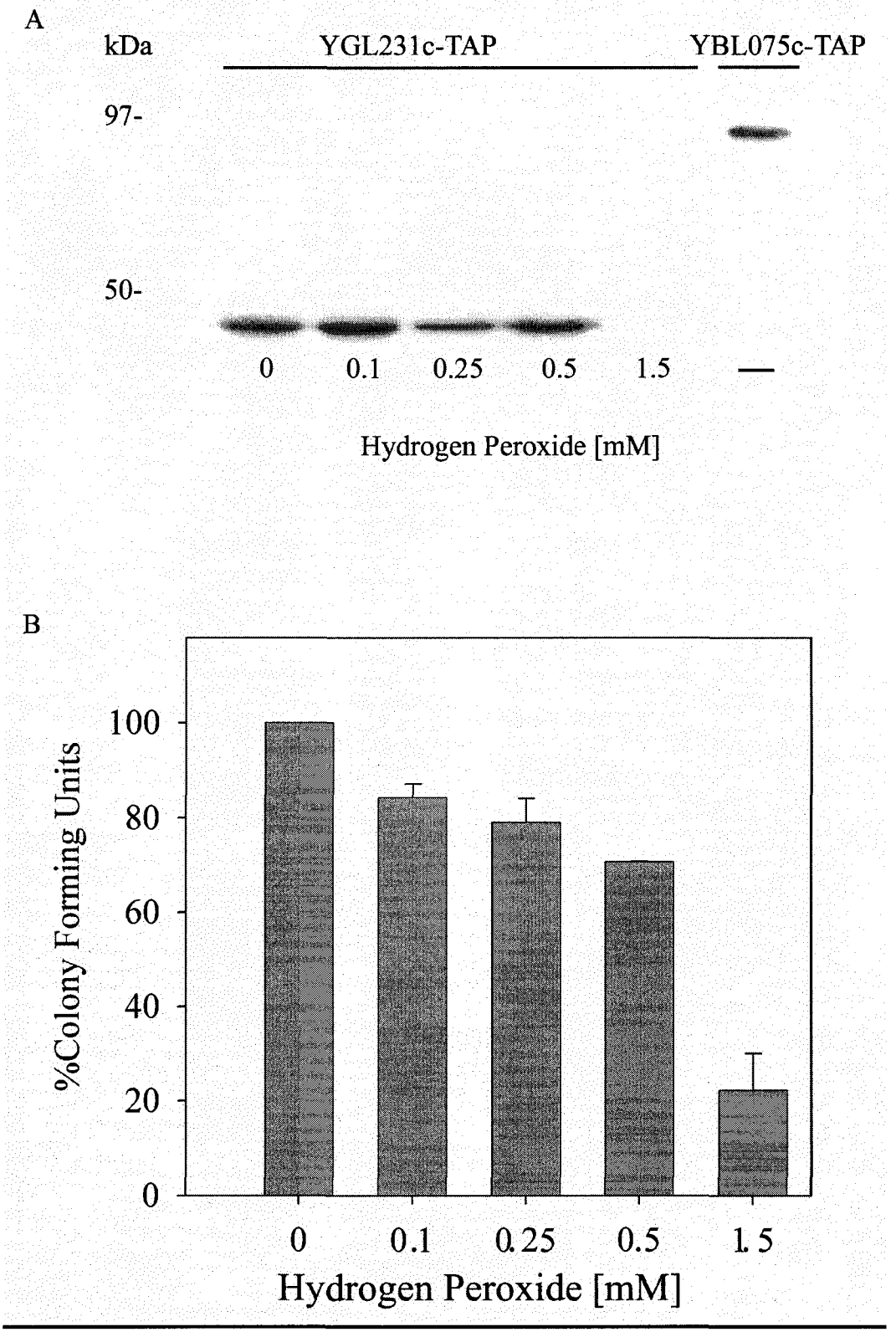
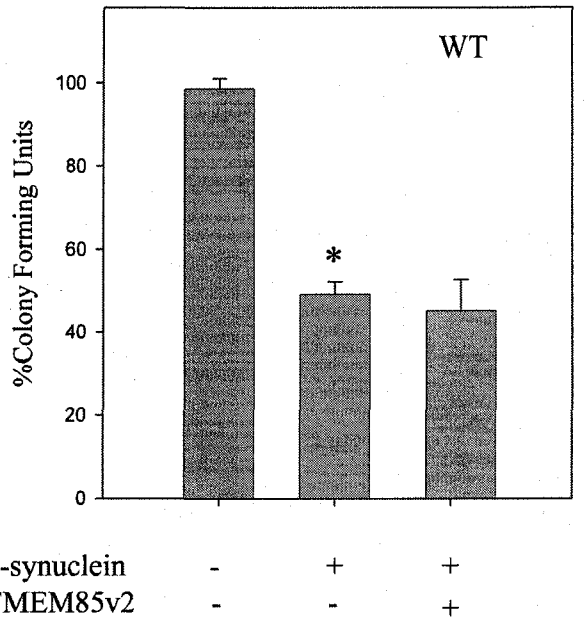

A
kDa
YGL231c-TAP
YBL075c-TAP
97-
50-
0
0.1
0.25
0.5
1.5
—
Hydrogen Peroxide [mM]
B
%Colony Forming Units
100
80
60
40
20
0
0
0.1
0.25
0.5
1.5
Hydrogen Peroxide [mM]

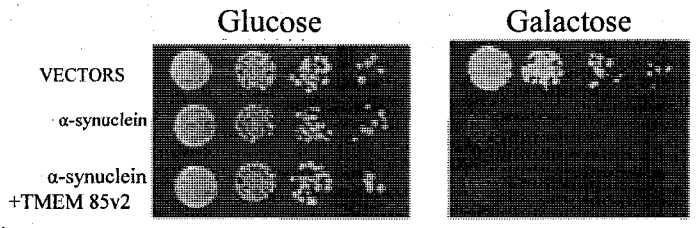
Figure 6. TMEM 85v2 does not protect against α-synuclein induced cell death.

(A)Wild type yeast cells were transformed with empty vector, α-synuclein expressing vector, and/or TMEM 85v2 expressing vector. Viability was determined for yeast expressing and not expressing the plasmids, and recorded as a percentage of viable cells observed in untreated cultures. Data are ± the standard error and represent a minimum of three independent experiments. "*" indicates significantly different than control cells harbouring α-synuclein, by student t-test ($p<0.001$) (B) Freshly saturated wild type yeast cultures transformed with empty vector, α-synuclein expressing vector, and/or TMEM 85v2 expressing vector were serially diluted. Aliquots (10μl) were spotted on glucose or galactose minimal media agar plates. (C) Deletion mutant yeast cells (YGL231cΔ) were transformed with empty vector, α-synuclein expressing vector, or TMEM 85v2 expressing vector. Viability was determined for yeast expressing the different plasmids, and the data is presented as the percentage of colony forming units that are observed. Data are ± the standard error and represent a minimum of three independent experiments.

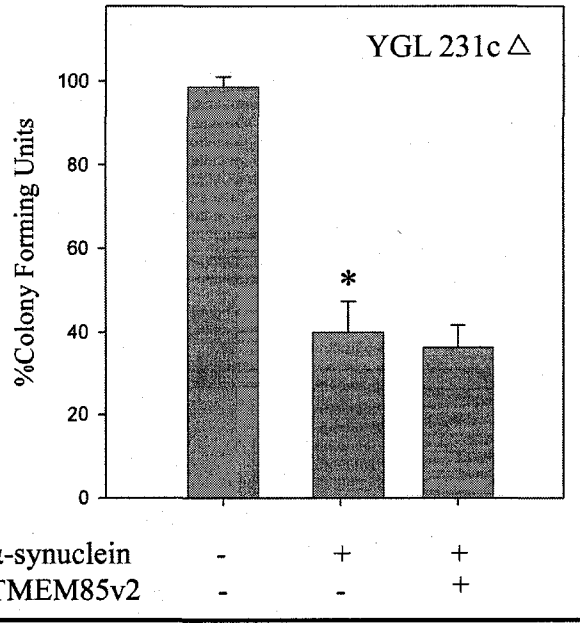
A


WT
%Colony Forming Units
100
80
60
40
20
0
*
α-synuclein - + +
TMEM85v2 - - +

B


Glucose
Galactose
VECTORS
α-synuclein
α-synuclein
+TMEM 85v2

C


YGL 231c △
%Colony Forming Units
100
80
60
40
20
0
*
α-synuclein - + +
TMEM85v2 - - +

Chapter 4: Discussion

As apoptosis is a key regulatory mechanism in the initiation and progression of disease, we sought to further elucidate factors involved in apoptosis. A number of successful screens of mammalian cDNA libraries have been carried out in yeast expressing heterologous Bax (reviewed in Osborn and Miller 2007). Results have yielded a number of genes whose expression is able to inhibit the lethal effects of Bax. We have previously shown that yeast can be a useful model system in the identification and characterization of novel mammalian anti-apoptotic genes (Yang, et al. 2006; Khoury, et al. 2007). In this study we attempted to characterize both the genomic organization and function of a novel anti- apoptotic gene called TMEM 85.

Based on analysis of the GenBank database and RT-PCR, we can conclude that TMEM 85 is an alternatively spliced gene that produces at least four distinct proteins (Fig. 2,3; Table 1,2). The gene is uniquely spliced likely utilizing the U12 spliceosome machinery. The frequency of splicing of U12 introns is significantly lower than that of U2 introns (Levine and Durbin 2001), indicating a less commonly expressed isoform. Exon 3 exhibits the odd ability to contain two exons, one for each type of machinery, nestled inside each other. While the splicing of this gene appears highly unusual, it has been observed elsewhere. The *Drosophila melanogaster prospero* gene contains a large intron that is spliced by the U12 machinery. However, within this intron are U2 type splice sites, generating a twintron (Scamborova, et al. 2004). The two splice sites compete with each other and are developmentally regulated.

We isolated the TMEM 85v2 splice variant in a previous screen of Bax suppressors from a human cardiac cDNA library (Yang, et al. 2006). The TMEM 85v2 protein has demonstrated an ability to protect cells from two different death inducing factors in yeast, Bax and hydrogen peroxide (Fig. 1). These factors, while exhibiting independent mechanisms of initiation of apoptosis, both lead to the production of reactive oxygen species within yeast (Madeo, et al. 2004). We found that TMEM 85v2 is not able to protect cells from apoptosis induced by ER stress. TMEM 85v2 was unable to protect yeast against three factors known to induce ER stress through independent mechanisms: tunicamycin though prevention of N-glycosylation, DTT through prevention of disulphide bonds, and α-synuclein through activation of the unfolded protein response (Fig, 1A, 6A, B, C). Although the yeast homologue of TMEM 85 is localized to the ER, it is likely that TMEM 85v2's anti-apoptotic role is not mediated through a reduction of ER stress. As well, TMEM 85v2 is not able to protect against apoptotic inducers which may bypass ROS as their main method of induction (Fig 1A). Therefore, it seems likely that TMEM 85v2 is able to mediate its anti-apoptotic effects by preventing apoptosis due to ROS.

We were unable to show any protective effect of TMEM 85v2 against α-synuclein mediated cell death (Fig. 6). It is possible that TMEM 85 is able to enhance cell survival under these conditions, but that our assay was not sensitive enough to detect it. The toxicity of α-synuclein increases linearly with increasing concentration (Outeiro and Lindquist 2003). The expressed levels of α-synuclein may have been too high for any anti-apoptotic factor to successfully rescue a cell.

To conclusively state no protective function exists, yeast need to be exposed to several increasing concentrations of α-synuclein.

The role of YGL231c as an endogenously expressed anti-apoptotic protein was explored through analysis of the deletion mutant. Yeast lacking YGL231c do not show increased apoptotic susceptibility in the presence of both low and high levels of hydrogen peroxide (Fig. 4B). Therefore, YGL231c is not a necessary component of the anti-apoptotic response in yeast. The overexpression of the YGL231c protein did nevertheless serve to protect the cells from hydrogen peroxide induced apoptosis (Fig 4C), indicating that YGL231c is able to exhibit an anti-apoptotic function. Thus, while YGL231c is not necessary for cell survival it is able to increase resistance to apoptotic stimuli. It is likely that other regulatory pathways exist in yeast that can compensate for the loss of YGL23c. As YGL231c shows homology to TMEM 85v1, it is also possible that TMEM 85v1 serves an anti-apoptotic function in humans. Our attempts to assess the anti-apoptotic potential of TMEM 85v1 were thwarted by the inability to obtain stable clones of the TMEM 85v1 PCR products.

As TMEM 85v2 is a truncated and C-terminally modified version of v1, we investigated the possibility that TMEM 85v2 was able to mediate its anti-apoptotic effects through interaction with v1 either antagonistically or collaboratively. TMEM 85v2 was able to enhance cell survival even with the loss of YGL231c in the deletion mutant (Fig.4C). Therefore the anti-apoptotic function of the two proteins likely occurs independently of one and other. While we have provided some functional characterization of two of the proteins, two,

TMEM 84v3 and v4, remain unknown. The anti-apoptotic character of each of the remaining proteins awaits further investigation. Future work should also investigate the role each isoform plays in the activity of the other. Different isoforms of the same gene often display opposite or directly antagonist functions (Hardy and O'Neill 2004).

We also used a yeast strain that expresses chromosomally TAP-tagged YGL231c to investigate endogenous regulation of the gene. Western blot analysis indicated that there was no up-regulation of the gene under our conditions of hydrogen peroxide induced stress (Fig. 5). It is possible that YGL231c is always expressed at a high level in yeast. It may be a constitutively expressed non-specific anti-apoptotic factor, such as a free radical scavenger. In spite of any speculation, analysis of the different TMEM 85 protein sequences does not give any clues as to the potential function of the proteins.

While few conclusions can be made about how TMEM 85v2 mediates its anti-apoptotic function, we have demonstrated that it is able to protect yeast against ROS induced apoptosis (Fig. 1). We provide evidence that TMEM 85 gene consists of five exons, and that it is spliced into four distinct transcripts and proteins (Fig.2,3; Table 1,2). We have demonstrated that two of the isoforms exhibit anti-apoptotic functions in yeast and that this function does not require both transcripts to occur (Fig. 4). Identification and characterization of novel anti-apoptotic sequences, like TMEM 85, is necessary to develop a clearer understanding of the process involved in regulating apoptosis. Only once the

process is elucidated can real progress be made in developing disease treatments that can target aberrantly expressed proteins involved in apoptosis.

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