CALCIUM DEPENDENT REGULATORY MECHANISM IN WOLFRAM SYNDROME

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Calcium dependent regulatory mechanism in Wolfram syndrome

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ABSTRACT

Wolfram syndrome is a genetic disorder characterized by diabetes and neurodegeneration. Two causative genes have been identified so far, *WFS1* and *WFS2*, both encoding endoplasmic reticulum (ER) localized transmembrane proteins. Since WFS1 is involved in the ER stress pathway, Wolfram syndrome is considered an ER disease. Despite the underlying importance of ER dysfunction in Wolfram syndrome, the molecular mechanism linking ER to the death of β cells and neurons has not been elucidated.

The endoplasmic reticulum (ER) is an organelle that forms a network of enclosed sacs and tubes that connect the nuclear membrane and other organelles including Golgi and mitochondria. ER plays critical functions in protein folding, protein transport, lipid metabolism, and calcium regulation. Dysregulation of ER function disrupts normal cell metabolism and activates an array of anti-survival pathways, eventually leading to disease state.

Here we show that calpain is involved in both prototypes of Wolfram syndrome. Calpain 2 activity is negatively regulated by WFS2 protein, and hyper-activation of calpain 2 by WFS2-knockdown leads to cell death. Calpain hyper-activation is also present in WFS1 loss of function cells due to the high cytosolic calcium. Extensive calpain activation exists in the Wolfram syndrome mouse model as well as in patient cells. A compound screen targeting ER homeostasis reveals that dantrolene, a ryanodine receptor inhibitor, can prevent cell death in cell models of Wolfram syndrome. Our

results demonstrate that the pathway leading to calpain activation provides potential therapeutic targets for Wolfram syndrome and other ER diseases.

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LIST OF ABBREVIATIONS

Abbreviation or symbol	**Term**
ALP	alkaline phosphatase
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
ATG5	autophagy protein 5
ATP	adenosine triphosphate
Bcl-2	B-cell lymphoma 2
BiP	immunoglobulin heavy chain-binding protein
BDNF	brain-derived neurotrophic factor
cAMP	cyclic adenosine monophosphate
CAPN1	calpain 1 catalytic subunit
CAPN2	calpain 2 catalytic subunit
CAPN10	calcium-activated neutral protease 10
CAPNS1	calpain small subunit 1
CAPNS2	calpain small subunit 2
CHOP	C/EBP-homologous protein
MYC	Myc proto-oncogene protein
CX	cycloheximide
DAPI	4', 6-diamidino-2-phenylindole
DIDMOAD	diabetes insipidus, diabetes mellitus, optic atrophy and deafness
DHA	docosahexaenoic acid
DMEM	Dulbecco’s modification of Eagle’s medium
E3	ubiquitin ligase

EDEM1	ER degradation-enhancing alpha-mannosidase-like 1
eIF2a	eukaryotic translation initiation factor 2, α subunit
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
FDA	Food and Drug Administration
GDNF	glia cell-derived neurotrophic factor
GLP-1	glucagon-like peptide 1
GSH	glutathione
GSIS	glucose stimulated insulin secretion
GST	glutathione S-transferase
HRD1	HMG-CoA reductase degradation protein 1
IGF-1	insulin-like growth factor 1
IP3	inositol trisphosphate
IP3R	inositol trisphosphate receptor
IPGTT	intraperitoneal glucose tolerance test
iPSCs	inducible pluripotent stem cells
IRE1	inositol-requiring enzyme 1
JNK	c-Jun N-terminal kinase
Kif4	chromosome-associated kinesin
MALDI-TOF	matrix assisted laser desorption ionization time-of-flight
MBP	myelin basic protein
MEF	mouse embryonic fibroblast
mitoNEET	CDGSH iron sulfur domain 1 protein
MRI	magnetic resonance imaging

NO	nitric oxide
OCT4	octamer-binding transcription factor 4
PARP	poly ADP ribose polymerase
PBA	4-phenylbutyrate
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PEK	pancreatic eIF-2α kinase
PERK	PKR-like ER kinase
PS1	presenilin 1
PS2	presenilin 2
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	media from Roswell Park Memorial Institute
RYR	ryanodine receptor
S1P	site 1 protease
S2P	site 2 protease
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SER	smooth endoplasmic reticulum
SERCA	sarcoplasmic endoplasmic reticulum calcium ATPase
shRNA	small hairpin RNA
siRNA	small interfering RNA

SSEA-4	stage-specific embryonic antigen-4
SOD1	superoxide dismutase 1
SOX2	sex determining region Y-box 2
T1D	type 1 diabetes
T2D	type 2 diabetes
TG	thapsigargin
TH	tyrosine hydroxylase
TM	tunicamycin
TRA-1	tumor rejection antigen 1
TUDCA	tauroursodeoxycholic acid
TUJ1	neuron-specific class III beta-tubulin
Ub	ubiquitin
UPR	unfolded protein response
UT	untreated
WFS1	Wolfram syndrome 1
WFS2	Wolfram syndrome 2
WT	wild type
XBP1	X-box binding protein 1

PREFACE

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CHAPTER I

INTRODUCTION

1.1 Diabetes and Wolfram syndrome

Diabetes mellitus is a metabolic disease affecting 382 million people worldwide and 28.6 million people in the United States. Each year, 1.5 to 5.1 million deaths are cause by diabetes mellitus worldwide, making it the 8th leading cause of death (World Health Organization, 2014). Diabetes is a group of diseases featured by patients experiencing abnormally high level of glucose over a prolonged period of time. In healthy population, glucose is generated after food absorption or breakdown of glycogen and other non-carbohydrate storage and is quickly uptaken by tissues such as liver, adipose tissue and muscles under the regulation of insulin. However, diabetic patients have defect in glucose uptake, resulting in elevated blood glucose level, a status called hyperglycemia. Symptoms of diabetes include excessive thirst, hunger, frequent urination, and weight loss. If left untreated, other complications may occur, such as eye problems, kidney failure, leg and foot pain, weakened immune system and nerve damage (Amos, 1997; Bell & Polonsky, 2001).

Insulin is a hormone release by pancreatic β cells, which are located at the islets of Langerhans in the pancreas. It ensures the blood glucose level homeostasis through binding to the cell surface insulin receptors and stimulating uptake of blood glucose into tissue cells. From there, glucose will be either consumed as an energy source or

converted for storage. In incidence of insulin production impairment or insulin resistance (inefficient cellular response to insulin), excess glucose cannot be absorbed (Muoio & Newgard, 2008). If blood glucose level is constantly high, many diabetic complications will occur. On the other hand, when blood glucose drops, insulin secretion decreases and secretion of another hormone by the pancreatic alpha cells—glucagon will be released leading to the inhibition of glucose absorption and breakdown of glycogen to increase blood glucose levels.

1.1.1 Major types of diabetes

There are two major forms of diabetes, Type 1 diabetes and Type 2 diabetes.

Type 1 diabetes (Diabetes mellitus type 1 or T1D) accounts for five to ten percent of all diabetic patients. It is an autoimmune disease in which pancreatic β cells are attacked and eliminated (Bluestone *et al*, 2010). Due to the importance of insulin in glucose transport and metabolism, T1D patients have elevated blood and urine glucose levels. Necessary treatment for the disease is insulin injection.

Type 2 diabetes (Diabetes mellitus type 2 or T2D) usually occurs among adults. About 90 percent of diabetic patients are T2D. Symptoms include hyperglycemia, insulin resistance and partial loss of pancreatic β cells. The cause of the disease is more complicated than T1D, it is a combination of genetics and environmental factors. The top risk factor for T2D is obesity, followed by diet, pregnancy, aging and gender (Kahn, 1998). Unlike T1D, T2D patient still have residual pancreatic βeta cells that can secrete insulin. These cells are usually overwhelmed by excessive demand of insulin production

due to the high blood glucose level, leading to cellular stress and eventually reduction of β cell mass. T2D patients also develop insulin resistance, in which case, glucose uptake by corresponding tissues is not sensitive to insulin regulation. A major concern in the field is how to improve the insulin sensitivity in T2D.

1.1.2 Wolfram syndrome

Wolfram syndrome is a rare autosomal recessive disease characterized by diabetes mellitus and optic atrophy usually during childhood or early adulthood. The prevalence of Wolfram is 1 in 770,000, with a carrier frequency of 1 in 354. It is also called DIDMOAD disease (Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy and Deafness) (Barrett *et al*, 1995). Diabetes is the first manifestation of Wolfram syndrome, occurring around age 6, followed by optic atrophy around age 11. Patients may also develop diabetes insipidus and deafness. Life expectancy of Wolfram syndrome is 30-40 years. Neurological disorders appear in most of the patients, usually starting from adulthood, symptoms include ataxia and depression. Magnetic resonance imaging (MRI) showed that patients have thinning of brain stem and atrophy in the cerebellum, which gives explanation for the neural disorder seen in patients. (Figure 1.1) There are two genetics forms of Wolfram syndrome, Wolfram syndrome 1 and Wolfram syndrome 2. Wolfram syndrome 1 composes more than 90 percent of all Wolfram syndrome cases.

Wolfram syndrome 1 patients have mutations in the *WFS1* gene, which encodes a protein called wolframin. Wolframin is a 100 KDa protein with 9 transmembrane segments located at the endoplasmic reticulum (ER). Despite being ubiquitously

expressed, WFS1 level is especially high in pancreas and brain. Previously WFS1 has been well characterized in pancreatic β cells, showing it to be important for ER stress response. Pancreatic β cells with mutation in *WFS1* gene have chronic high basal unfolded protein response (UPR) levels; they cannot recruit E3 ubiquitin ligase—HRD1 to degrade activated ATF6 protein, leading to intolerable ER stress (Fonseca *et al*, 2010). WFS1 deficient cells also have impairment in insulin synthesis and are vulnerable to ER stress inducers. We used mice that have WFS1 specifically knocked out in pancreatic β cell (Ishihara *et al*, 2004; Riggs *et al*, 2005). These mice showed high UPR marker expression and defect in insulin production. However, these animals only developed impaired glucose stimulated insulin secretion and a reduction in β cell mass, but not insulin resistance. WFS1 has also been shown to be involved in calcium regulation, studies showed the critical role of WFS1 in retention of ER calcium level (Osman *et al*, 2003). Some studies even proposed WFS1 as a new calcium channel. So far, over 100 different mutations have been found in the *WFS1* gene among Wolfram syndrome patients, including missense, nonsense and deletion mutations. These mutations concentrate mainly at transmembrane domains, N-terminal and the C-terminal of the protein. (Figure 1.2) It is also interesting to notice that 8.31% of Wolfram syndrome patients have only one *WFS1* mutant allele (de Heredia *et al*, 2013). Therefore, in some cases of Wolfram syndrome, *WFS1* mutants are autosomal dominant (Hansen *et al*, 2005).

Wolfram syndrome 2 patients have mutations in the *WFS2* gene. *WFS2* (also known as CISD2, Miner1, NAF-1 and Eris) is a not as well characterized as *WFS1*. It

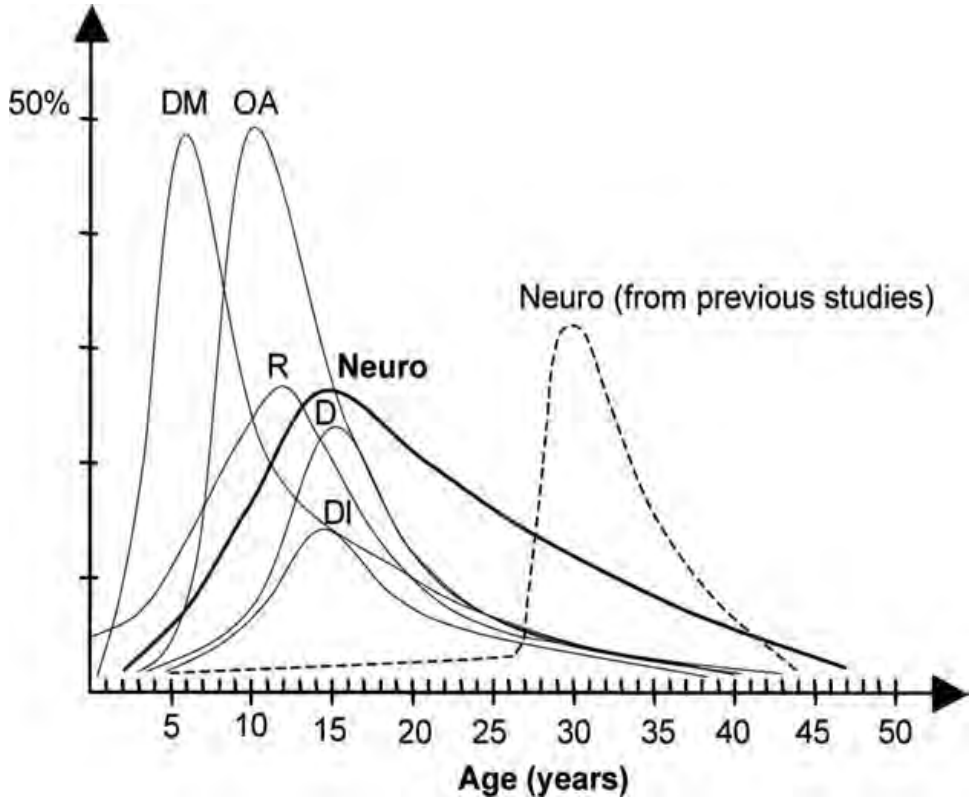


Figure 1.1 Clinical characteristics of Wolfram syndrome patients. Adapted from Barrett and colleagues. The peak of each curve represents the median age of onset of the complications and the intersections with the x-axis represent the ranges. The dotted line corresponds to the median age of onset of neurologic complications in the Barrett's series. D = deafness; DI = diabetes insipidus; DM = diabetes mellitus; Neuro = neurologic complications; OA = optic atrophy; R = renal tract abnormalities (Chaussenot *et al*, 2011).

belongs to a protein family containing CDGSH iron-sulfur domain, another member of the family is mitoNEET, which has been identified as a target of the anti-diabetic drug—Pioglitazone (Paddock *et al*, 2007). Patients with homozygous *WFS2* mutation were first identified in three consanguineous Jordanian families with Wolfram syndrome (Amr *et al*, 2007a). So far, 2 types of mutations in the *WFS2* gene were discovered in Wolfram

syndrome patients. One is a point mutation in exon 2, the other type is a intragenic deletion in exon 2 (Mazzillo *et al*, 2014). Compared to patients with *WFS1* mutations, WFS2 patients also develop diabetes and neuronal disorders. In addition, they exhibit clinical features such as bleeding tendency, defective platelet aggregation and peptic-ulcer disease. Recently, WFS2 whole body knockout mice were generated. WFS2 knockout mice exhibit shorter life span, glucose intolerance, neuron degeneration and muscle atrophy, whereas WFS2 knockin mice have longer lifespan compared to littermate controls (Chen *et al*, 2009b, 2009a). It has also been shown that WFS2 is involved in Belkin 1-dependent autophagy by binding ER BCL2 protein (Chang *et al*, 2010). WFS2 patients compose less than 10 percent of Wolfram syndrome population, due to the rareness of Wolfram syndrome 2, there is no patient study thus far. Therefore, understanding the molecular function of WFS2 may help establish a treatment for Wolfram syndrome and related common diseases. However, very little is known about the WFS2 protein.

There is currently no treatment for Wolfram syndrome. Many Wolfram syndrome patients are treated as diabetic patients and this helps maintain their blood glucose levels. However, other complications cannot be controlled, such as neural complications. Central apnea is the major cause of death in Wolfram syndrome. It has been shown that neurologic complications occurred in over 50% of the patients with a median age of 15 (Chaussenot *et al*, 2011). The top 3 neurologic complications in Wolfram syndrome are cerebellar ataxia, peripheral neuropathy and cognitive impairment. These features suggest that there are neurodegeneration and neurodevelopmental

abnormalities in the patients, which is confirmed by MRI study of the human brain. MRI revealed atrophy in cerebellum, brain stem and cerebral in the majority of the patients, while optic atrophy, thinning of hypothalamus and infundibulum, abnormality of white matter, cortical abnormalities were also noticed in some patients (Hardy *et al*, 1999; Chaussenot *et al*, 2011). Due to the neuronal pathological features of the disease, understanding the cause of neuronal cell death in Wolfram syndrome became an urgent demand in the field.

1.2 ER homeostasis

Endoplasmic reticulum is an organelle in eukaryotic cells that forms a network connecting the nuclear membrane and other organelles such as Golgi and mitochondria. It appears as membrane folded into enclosed sacs or tubes. There are two kinds of ER, rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER). The rough ER gets its name because of the ribosomes scattered on its cytosolic face while smooth ER does not have ribosomes binding to it. Endoplasmic reticulum functions in protein folding, protein transport, lipid metabolism, and calcium regulation (Figure 1.3) (Burdakov *et al*, 2005; Hebert & Molinari, 2007; Fu *et al*, 2012). The environment of the ER is very special compared to other cellular compartments. It has a high calcium concentration (about 10,000 fold higher calcium in the ER compared to cytosol), which makes it the ideal location for calcium dependent proteins (Clapham, 2007). The high calcium level in the ER can also function as a part of signal cascades operated by secondary messengers. What's more, the ER lumen has an oxidative environment, which

is important for disulfide bond formation and proper protein folding. In addition, ER is the major location for protein modification. This is especially critical for secretory proteins, which need to be tagged before transported to the right locations.

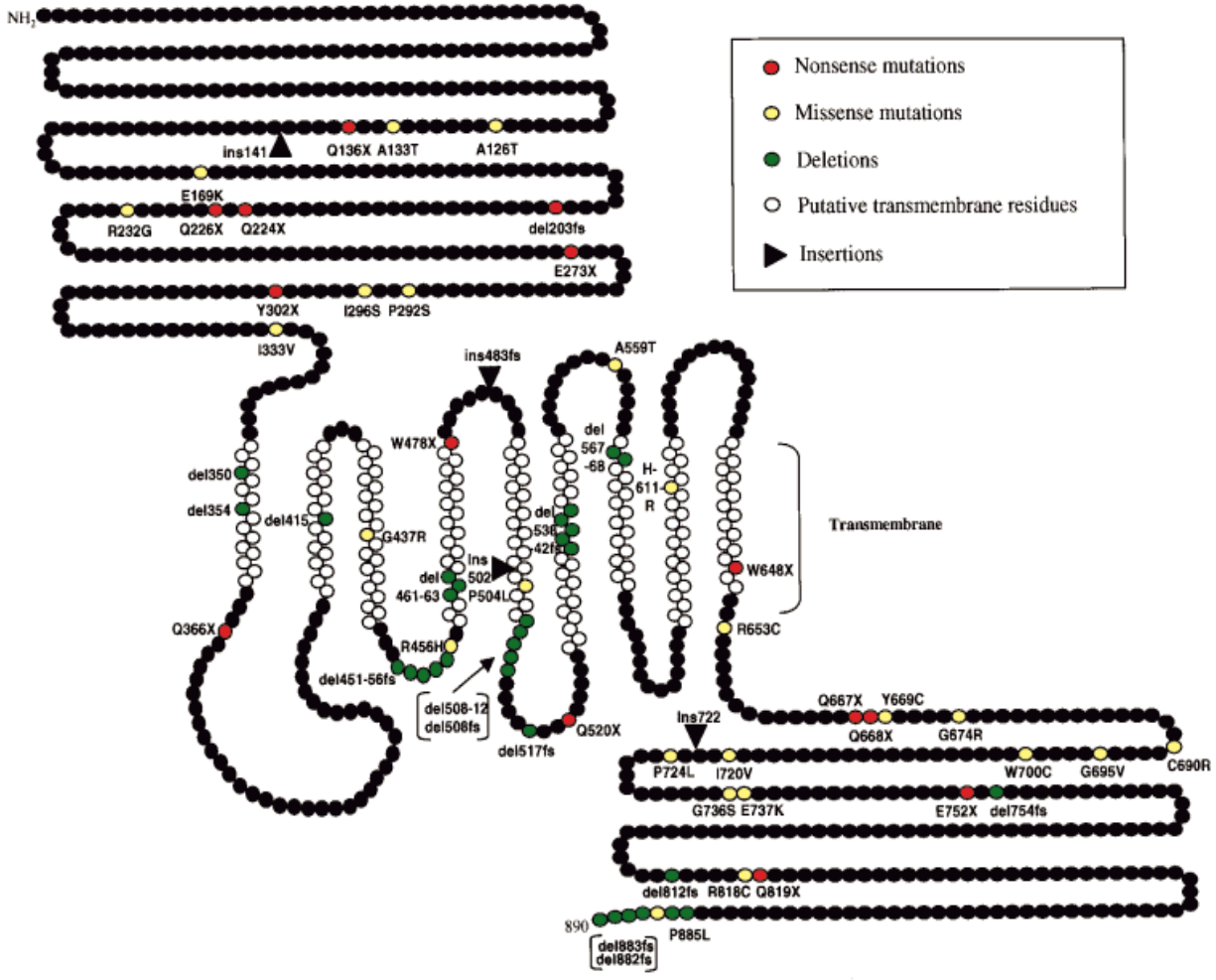


Figure 1.2 Disease mutations in WFS1 protein. WFS1 is an ER transmembrane protein. Mutations in WFS1 gene includes missense, nonsense and deletion mutations. There is no special hot spot where mutations occur (Reproduced from Farhat Khanim,1 Jeremy Kirk,2 Farida Latif,1 and Timothy G. Barrett 2001).

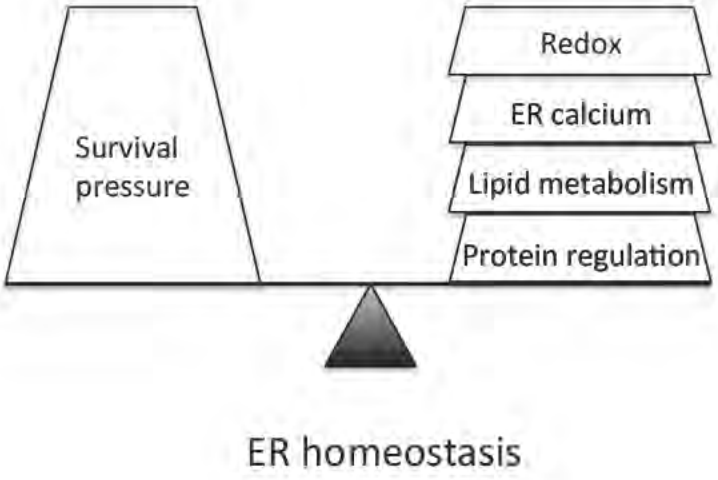


Figure 1.3 ER homeostasis. The endoplasmic reticulum plays a critical role in facilitating cells in our bodies to confront survival pressure through modulating redox state, ER calcium levels, lipid metabolism, and protein functions.

1.2.1 UPR and ER homeostasis

Proteins are biological work units that need to correctly fold into their three dimensional structure to function properly. One of the major functions of the endoplasmic reticulum is folding of newly synthesized proteins. The endoplasmic reticulum provides the oxidizing environment required for disulfide bond formation, and it also contains various protein chaperones that facilitate the folding of some proteins by lowering the energy barrier. To ensure that proteins are not overloaded and unfolded/misfolded proteins can be detected and removed, endoplasmic reticulum has a

precise signaling network to regulate the folding process called the unfolded protein response (UPR) (Ron & Walter, 2007; Kaufman *et al*, 2002).

Maintaining a balance between ER protein load and its folding capacity is critical for cell survival. However under certain circumstances, ER protein load exceeds its folding capacity, this will cause accumulation of unfolded/misfolded proteins in the ER, leading to ER stress. Eukaryotes developed a conserved pathway to counterbalance ER stress called the unfolded protein response. Under normal conditions, the UPR pathway ameliorates ER stress by suppressing global protein translation, accelerating the protein-folding process, and facilitating protein degradation. However, if the ER stress level is irreversible, the UPR will activate a different set of genes promoting cell death (Oslowski & Urano, 2011). Under ER stress conditions, the unfolded/misfolded proteins interact with ER localized chaperone—immunoglobin binding protein (BIP), releasing it from ER stress sensors, thus activating downstream signaling pathways. There are three ER stress sensors in the unfolded protein response pathway, IRE1, PERK and ATF6 (Figure 1.4).

IRE1 (inositol-requiring enzyme 1) is a serine/threonine kinase with endoribonuclease activity (Mori *et al*, 1993). It is an ER transmembrane protein conserved in all eukaryotes (Tirasophon *et al*, 1998). Upon activation, IRE1 alternatively splices XBP1 mRNA, removing a 26nt intron, resulting in the production of XBP1s (Yoshida *et al*, 1998, 2001). XBP1s would then regulate downstream UPR signaling including genes involved in the ER associated degradation (ERAD) process (Lee *et al*,

2003). Recently IRE1 was found to cleave many other mRNA transcripts in the ER in addition to XBP1. Degradation of these mRNA can reduce the translation load, which is beneficial for stress recovery (Han *et al*, 2009; Hollien & Weissman, 2006). When ER stress is irreversible, IRE1 would activate pro-apoptotic signal cascade such as the JNK signaling pathway (Urano, 2000).

PERK (PKR-like ER kinase) functions as an ER transmembrane protein kinase of the PEK family (Harding *et al*, 1999). Under ER stress condition, PERK activation induces phosphorylation of eIF2α (Kimball, 1999). This shuts down global protein translation, which helps reduce the protein load in the ER. At the same time, eIF2α phosphorylation activates transcription factor ATF4, which can increase production of molecular chaperones and enhance amino acid metabolism, therefore ameliorates the accumulation of unfolded proteins (Vattem & Wek, 2004). However, prolonged suppression of protein synthesis is fatal for cells. Thus, if ER homeostasis cannot be achieved, programed cell death pathways will be activated.

ATF6 (activating transcription factor 6) is another transcription factor that can sense ER stress (Yoshida *et al*, 1998). It is tethered to the ER by BiP protein (Shen *et al*, 2002). When ER stress occurs, BiP is released and ATF6 translocates to the Golgi where it is cleaved by proteases (S1P and S2P) into the processed form (Haze *et al*, 1999). Then, the processed ATF6 migrates into the nucleus and facilitates the transcription of downstream UPR genes. Targets of ATF6 include pro-survival genes involved in protein

folding and ERAD including *BiP*, *PDI*, and *EDEM1* as well as anti-survival genes like *CHOP* (Ma *et al*, 2002).

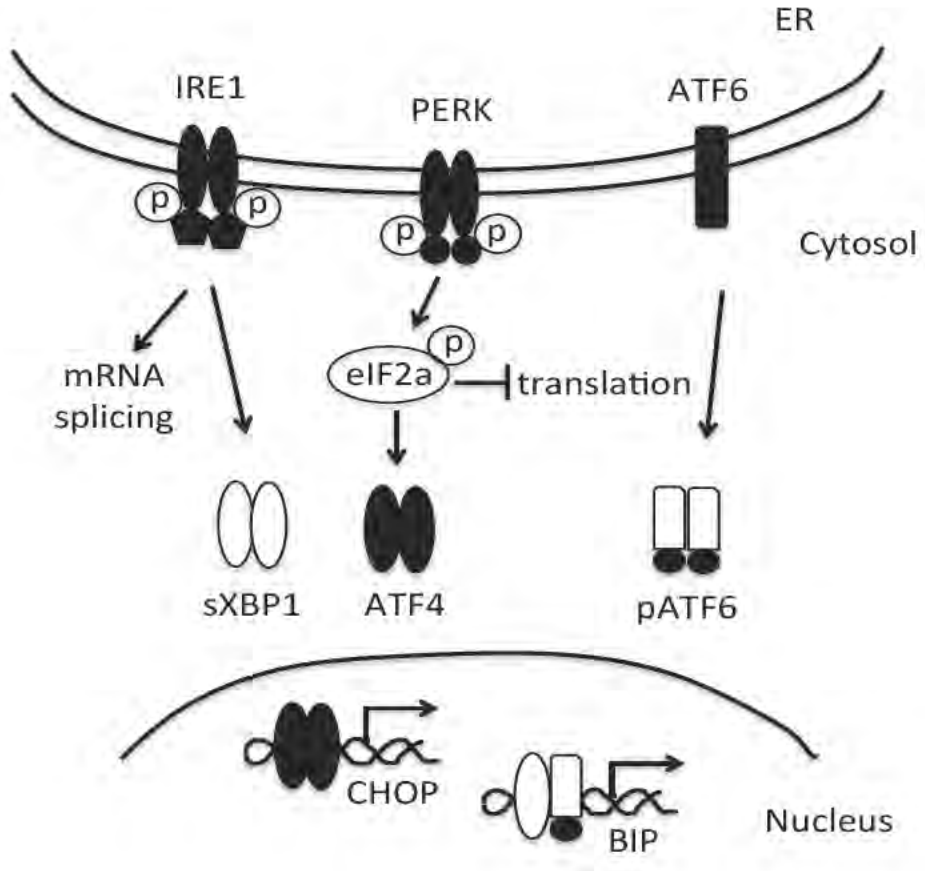


Figure 1.4 The unfolded protein response. Under ER stress conditions, three UPR sensors (IRE1, PERK, ATF6) will be activated and regulate downstream pathways to alleviate ER stress, promoting cell survival. However, under unresolvable ER stress conditions, the UPR sensors would induce the pro-apoptotic pathways leading to cell death.

1.2.2 Lipid metabolism and ER homeostasis

Lipid is mainly synthesized in the smooth endoplasmic reticulum. Because of the hydrophobic nature of lipids, they are synthesized in association with ER membrane. They are then transported to different parts of the cell. Certain cell types such as hepatocytes contain abundant smooth ER to adapt to the high demand of detoxification, removal of hydrophobic components, lipids processing and synthesis.

The ER is a membrane rich organelle where lipid composition can affect its membrane structure and membrane protein function (Fu *et al*, 2012). Treatment of cells with saturated fatty acid or cholesterol can induce acute ER stress which could be compensated by the UPR pathway (Borradaile *et al*, 2006; Wei *et al*, 2006; Feng *et al*, 2003). Chronic lipid overload is even more pathological. For example, in some obese patients, lipid overload causes lipotoxicity and perturbed ER lipid composition, leading to functional impairment of SERCA, a main calcium pump at the ER. Therefore, ER homeostasis will be disrupted. (Li *et al*, 2004; Fu *et al*, 2011)

1.2.3 Calcium regulation and ER homeostasis

Calcium is an important signaling molecule involved in all aspects of cellular functions including proliferation, trafficking, secretion, and apoptosis. Calcium is stored in the ER and mitochondria, and maintained at low concentration in the cytosol (10-100 nM) by calcium pumps that actively pump calcium from the cytosol into the ER (Figure 1.5). The most active calcium pump located at the ER is sarco/endoplasmic reticulum calcium-ATPase (SERCA), and SERCA pumps calcium into the ER at the cost of ATP

(Moore *et al*, 1975). The ER also contains several calcium channels that release calcium into the cytosol, including inositol 1,4,5-trisphosphate receptor (IP3R) and ryanodine receptor (RYR). It is critical to maintain high calcium concentration in the ER since many enzymes including molecular chaperones require calcium for proper function (Michalak *et al*, 2002). Additionally, low cytosolic calcium is also important to keep calcium mediated signaling events inactivate under normal circumstances (Berridge *et al*, 2003). So once calcium is released into the cytosol, the large gradient wave of calcium can trigger a wide variety of signaling cascades.

SERCA (sarco/endoplasmic reticulum calcium-ATPase) is a calcium pump located at the ER. There are three genes encoding SERCA, *ATPA1-3*, whereby, transcription of the gene results in multiple splicing variants. The major form of ER calcium –ATPase in non-muscle tissue is SERCA2b. Inhibition of SERCA pump by thapsigargin activates the UPR pathway and induces ER stress.

IP3R (inositol 1, 4, 5-trisphosphate receptor) functions as a ubiquitously expressed calcium release channel located at the ER membrane. Binding of inositol trisphosphate (IP3) at the amino terminus of IP3R stimulates structural transition and channel opening at the carboxyl terminus. IP3R has various functions including cell proliferation, differentiation, and apoptosis.

RyR (ryanodine receptor) is a major calcium release channel at the ER membrane. Mammalian RyR exists in three different isoforms: RyR1 is mainly expressed in skeletal muscles; RyR2 is primarily expressed in the myocardium; RyR3 is expressed

widely but predominantly in the brain. RyR disruption occurs in many disease manifestations. The only existing prescription drug targeting RyR is dantrolene. Disturbance in ER calcium can perturb cellular events critically linked to cell survival.

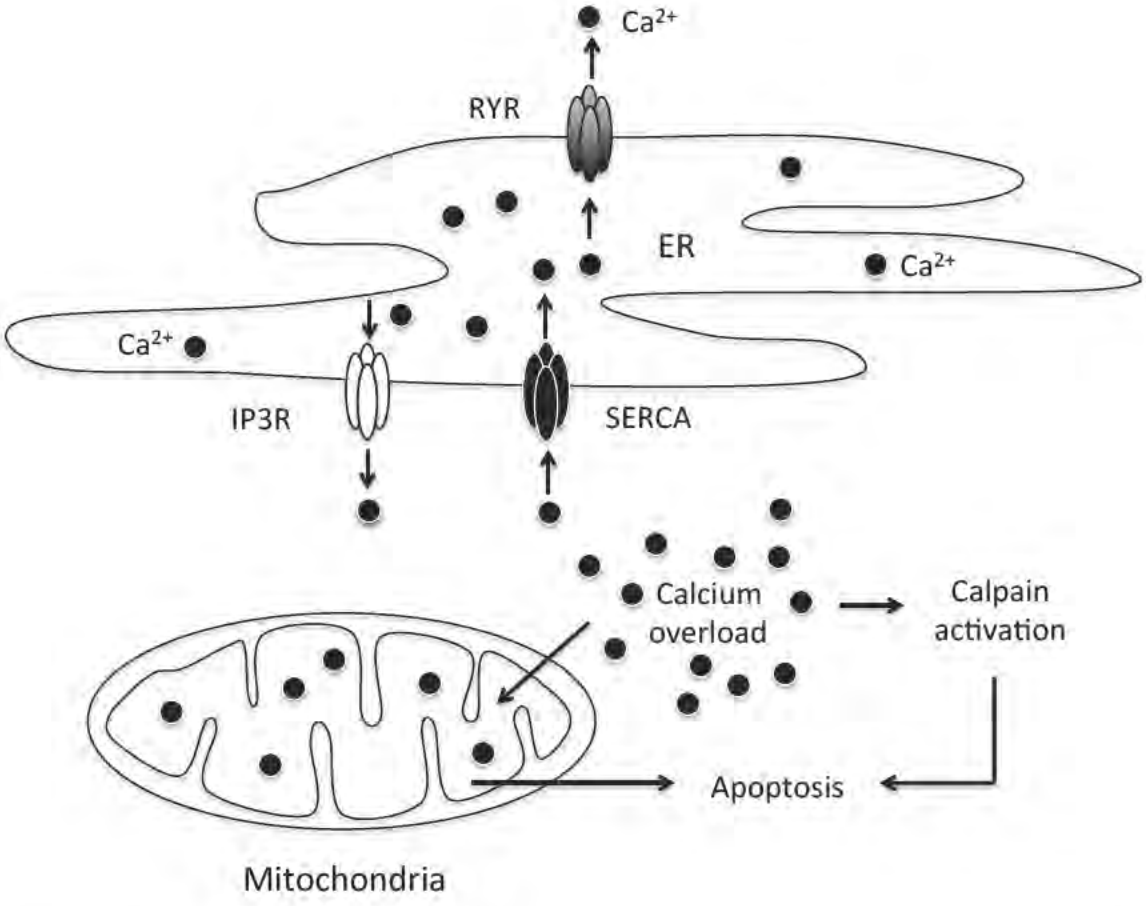


Figure 1.5 ER calcium channels. Calcium channels on the ER membrane include SERCA, IP3R and RYR. SERCA pumps calcium into the ER while IP3R and RYR releases calcium from the ER to cytosol. Regulation of cytosolic calcium level is critical for cell survival. High cytosolic calcium can directly lead to the activation of anti-survival protease—calpain or uptaken by mitochondria and cause high mitochondrial calcium levels, which in turn leads to apoptosis.

Leakage of ER calcium into the cytosol raises the cytosolic calcium level, which would trigger the mitochondria to work as a calcium buffering system and uptake excess calcium. Normally, calcium is an important modulator in mitochondria acting as a stimuli for ATP synthesis. However, mitochondria calcium overload can enhance ROS generation, increase permeability of transition pores and trigger cytochrome c release leading to apoptosis (Brookes *et al*, 2004). High cytosolic calcium could also activate anti-survival calcium dependent proteases such as calpains. Once activated, these enzymes cleave a vast variety of substrates, damaging cellular structure and function (Zatz & Starling, 2005; Huang & Wang, 2001).

1.2.4 ER homeostasis and disease

1.2.4.1 UPR and diseases

In T1D, it was proposed that β cell death is induced by overproduction of NO, and the pro-apoptotic molecule--NO is regulated by CHOP, an ER stress pro-apoptotic transcription factor. In a genetic type of diabetic mouse model (Oyadomari *et al*, 2001), Akita mice have a high ER stress level. What's more, homozygous deletion of CHOP significantly delays the disease onset (Oyadomari *et al*, 2002). In T2D mouse models, when the mice were fed with high fat diet, signs of ER stress have been found in islets, liver and adipose tissue (Ozcan *et al*, 2004). Ablation of CHOP can protect against apoptosis and preserve β cell mass (Oyadomari *et al*, 2002; Song *et al*, 2008). In addition, molecular chaperons such as PBA, TUDCA and curcumin have been proven to improve

protein-folding capacity and ameliorate ER stress, thus improving β cell survival, insulin sensitivity and glucose homeostasis in diabetic mice (Ozcan *et al*, 2006).

WFS1, the causative gene for Wolfram syndrome, is part of the UPR pathway. In a diabetic mouse model of Wolfram syndrome, E3 ligase HRD1 mislocalization leads to ATP6 hyper-activation in pancreatic β cells. This eventually causes the loss of β cell mass and impairment of insulin production.

Another typical ER stress disease is Wolcott-Rallison syndrome (WRS). It is a rare, autosomal recessive disorder with infancy-onset diabetes, multiple epiphyseal dysplasia, osteopenia, mental retardation or developmental delay, and hepatic and renal dysfunction as main clinical findings. Patients with WRS have mutations in the *EIF2AK3* gene, which encodes the PERK protein (Delépine *et al*, 2000). This blocks the phosphorylation of eIF2α, which will further prevent β cell development and induces cell death.

In the case of Alzheimer's, PERK –eIF2α pathway is hyperactive in brain tissue, implying a relationship between ER stress and Alzheimer's disease (Unterberger *et al*, 2006). Mutations in the Parkin protein account for a small portion of Parkinson's disease. It is an E3 ubiquitin ligase and mutation of Parkin disrupts the ERAD system. In contrast, cells overexpressing Parkin are more resistant to ER stress induced cell death (Dawson & Dawson, 2003). Two percent of ALS cases have a mutation in the *SOD1* gene. Mutant SOD1 protein forms aggregates that induce the UPR pathway through exhausting the proteasome activity in a failed attempt to degrade them. Moreover, SOD1

mutants could also directly bind the ERAD machinery, tipping the balance between protein production and degradation (Nishitoh *et al*, 2008). In another neurodegenerative disease, the prion disease, pathological form of the prion protein PrP^{sc} can induce calcium depletion in the ER upon treatment, which results in the activation of the UPR. Molecular chaperones have been reported to be elevated in brains of these patients. This could be confirmed in cell culture that prion treated cells showed induction of ER stress markers (Hetz *et al*, 2005). A treatment strategy for prion disease is modulating ER stress levels by augmenting the expression of protein chaperones.

Reports show that proteins associated with the UPR are upregulated in rapidly growing tumors. These upregulated genes provide a protective effect in cancer cells (Shuda *et al*, 2003; Jamora *et al*, 1996; Bi *et al*, 2005). It is important we determine a way to inhibit the protective branches and activate the anti-survival branches of UPR in cancer cells for treatment purposes. Supporting this concept, chemical inhibitors of BiP are being investigated as a potential cancer drug target (Lee, 2007). At the same time, protein synthesis and modification is more active in cancer cells requiring massive protein regulatory machineries. Thus, by inhibiting protein regulation pathways such as the ERAD, anti-survival branches of the UPR could be activated. Proteasome inhibitors that can induce ER stress and promote cytotoxic effect in cancer cells are being studied for clinical purposes. Bortezomib, a proteasome inhibitor has been used as a treatment for multiple myeloma (Nawrocki *et al*, 2005).

1.2.4.2 ER redox disease

The endoplasmic reticulum has an irreplaceable role in protein folding and trafficking, which is extremely sensitive to ER luminal environment. Subtle changes in the redox state could impair ER function tremendously. Alterations of the components of ER redox system can cause redox change, including hypoxia, impairment in oxidative protein folding, deletion of glutathione (GSH), and decreased activity of folding machinery component PDI and production of ROS (Holtz *et al*, 2006; Bass *et al*, 2004; Uehara *et al*, 2006). Reactive oxygen species (ROS) production is a natural part of cell metabolism. However, if not removed properly, accumulation of ROS could be harmful for the cells. ROS production occurs in the cytosol and many other organelles including ER. Accumulation of ROS in the ER can lead to ER stress, which in turn could induce even more ROS production (Haynes *et al*, 2004). Diseases associated with redox change have been reported over the last few decades. In Parkinson's disease, S-nitrosylation of PDI and parkin imply ROS presence in ER lumen of neurons (Nakamura & Lipton, 2008). GSH depletion and massive oxidation were also seen in Parkinson's disease (Danielson & Andersen, 2008). Some observations suggest that progression of Prion disease is also related to ER redox state. Conversion of the soluble prion proteins into insoluble form is the key event in developing the disease. Reduced environment in the ER facilitates the production of insoluble prion proteins, thus increases the risk of developing Prion disease (Capellari *et al*, 1999; Tuzi *et al*, 2008).

1.2.4.3 Dysregulation of ER calcium diseases status

ER homeostasis is critical for cell survival. Abnormal cytosolic calcium caused by disturbance of calcium channels, calcium transporters, calcium binding proteins or calcium channels can induce disease manifestations.

In the case of T1D, β cells are destroyed by NO overproduction. NO depletes ER calcium by down-regulating SERCA2b production through inhibition of Sp1 transcription factor (Pierre Pirot, Alessandra K Cardozo, 2008; Cardozo *et al*, 2005). NO can also form peroxynitrite in the presence of superoxide, which inhibits SERCA and activates RyR channels (Grover *et al*, 2003; Xu, 1998). Moreover, cytokines in T1D can up-regulate BH3-only proteins, thus leading to ER stress and calcium depletion (Gurzov *et al*, 2009). Additional evidence showing the importance of calcium homeostasis in T1D is that an IP3R3 polymorphism is associated with the disease (Roach *et al*, 2006).

T2D is also associated with dysregulated calcium homeostasis. Thapsigargin treatment depletes calcium in the ER and can cause insulin resistance (Ozcan *et al*, 2004). It has been shown that high glucose and free fatty acid treatment can cause ER calcium depletion as well as activate UPR pathways in pancreatic β cells (Eizirik *et al*, 2008). One possible mechanism for the ER calcium depletion is through a decreased level or activity of SERCA (Evans-Molina *et al*, 2009; Cunha *et al*, 2008). In a genetic form of diabetes—Wolfram syndrome, a defect in maintenance of ER calcium occurs and triggers UPR pathway leading to apoptosis (Hara *et al*, 2014).

Calcium disruption also takes place in the case of neuronal disorders. Brain ischemia induces ER calcium depletion into the cytosol (Verkhratsky, 2005). Increased

cytosolic calcium enhances NO synthesis, which can inhibit SERCA activity (Moncada & Erusalimsky, 2002; Doutheil *et al*, 2000). Neural ischemia can also activate RyR activity by induction of NO, reactive oxygen species and calpain proteolysis (Bull *et al*, 2008; Rardon *et al*, 1990). Finally, there is strong evidence showing that in a genetic form of early onset Alzheimer's disease, the presenilins have a crucial role in modulating intracellular calcium signaling (Sepulveda-Falla *et al*, 2014; Bezprozvanny, 2013). ER calcium stores are overfilled as a consequence of presenilin mutations. This alteration in calcium homeostasis is a driving force for PS-1 and PS-2 Alzheimer's disease development.

1.3 Calpain

Calpains are calcium dependent cysteine proteases. They form heterodimers composed of a large catalytic subunit and a small regulatory subunit. There are 14 members of the human calpain family that have been identified as the catalytic subunit, together with two members of the regulatory subunit CAPNS1 and CAPNS2 (Suzuki *et al*, 2004; Vosler *et al*, 2008). Calpain 1 (also called u-calpain) and calpain 2 (also called m-calpain) are the best-characterized calpain family members; they share the same regulatory subunit (CAPNS1) but have distinct catalytic subunits CAPN1 and CAPN2. Activation of calpain requires translocation of the molecule from the cytosol to membranes. In the joint presence of high calcium and membrane phospholipids, calpain undergoes auto-hydrolysis to an active form. Activation of calpain must be tightly

regulated; dysregulation of calpain can cause excess cleavage or accumulation of substrate proteins leading to severe cell damage and pathological condition.

Calpains have been shown to participate in multiple activities including cell migration, cell cycle, neuron potentiation, necrosis, autophagy, and apoptosis. It was reported that calpain acted as a switch between autophagy and apoptosis by cleaving autophagy-related gene 5 (Atg5). (Yousefi *et al*, 2006) Cleaved Atg5 translocates to the mitochondria and triggers cytochrome c release and caspase activation, thus, inducing apoptosis. Hyper-activation of calpains is found in various diseases. In Alzheimer's disease, studies showed significant increase of calpain activity in brains of Alzheimer's disease patients. This may be due to enhanced intracellular calcium concentration induced by beta-amyloid or decreased calpastatin levels, which is an endogenous inhibitor of calpain (Saito *et al*, 1993; Ferreira & Bigio, 2011; Peterson & Goldman, 1986; Tsuji *et al*, 1998; Kelly *et al*, 2005; Kelly & Ferreira, 2006; Rao *et al*, 2008). In the case of type 2 diabetes, calpain 2 is hyper-activated, genetic screening for diabetes genes in Mexican-American and northern European populations revealed CAPN10 as a diabetes-susceptibility gene (Harris *et al*, 2006). Genetic variations of the CAPN10 gene are related to insulin resistance and elevated free fatty acids. Moreover, exposure of mouse islets to calpain inhibitor enhanced glucose stimulated insulin secretion (Sreenan *et al*, 2001). Hyper-activation of calpains induces cleavage of downstream substrates including many cell structural proteins, ion channels and enzymes, thus, disrupting cellular structure and impairing cellular function (Figure 1.6).

Calpain knockout mice have been generated as an animal model to study calpain diseases. CAPN1 knockout mice are viable and fertile but showed some defect in platelet aggregation (Azam *et al*, 2001). This might be due to functional compensation by CAPN2. However, CAPN2 knockout mice are embryonically lethal, which indicates

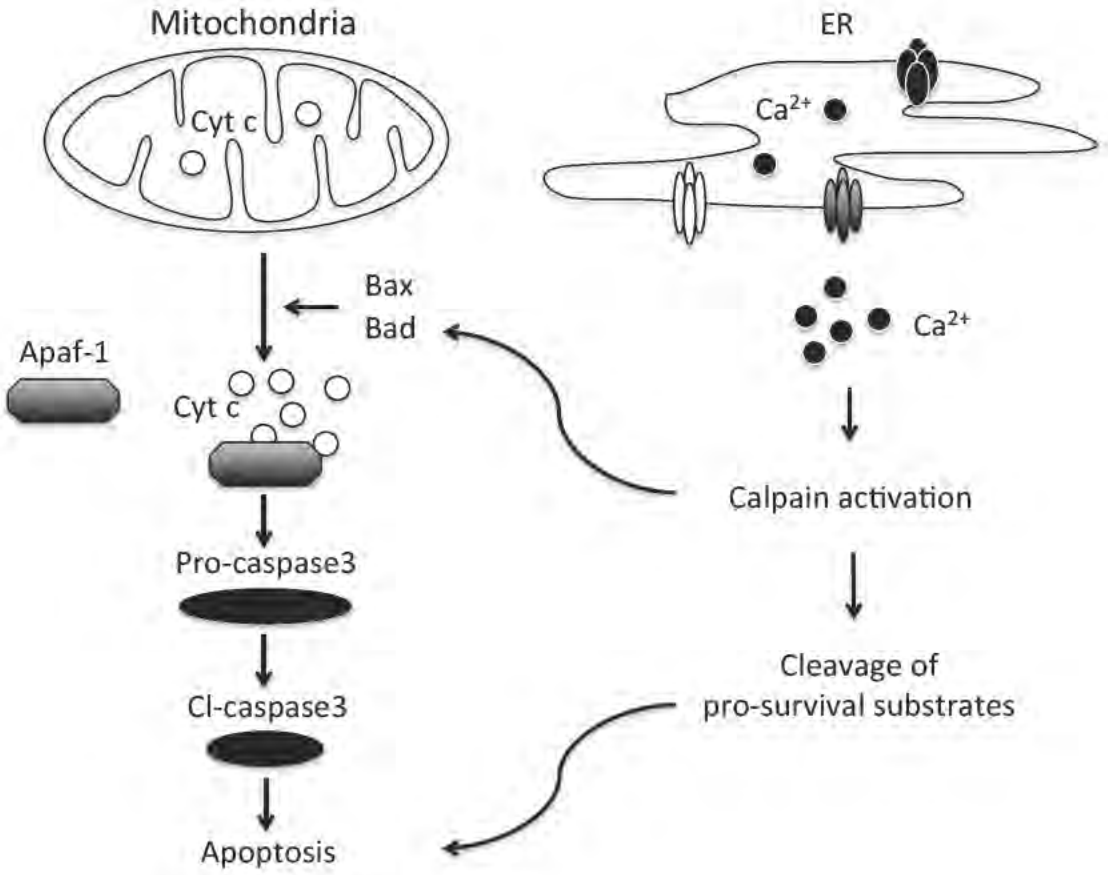


Figure 1.6 Calpain activation leads to apoptosis. Depletion of ER calcium into the cytosol activates calcium dependent proteases such as calpains. Calpains can either directly cleave pro-survival substrates or regulate BCL2 family members leading to the release of cytochrome c and apoptosis.

CAPN2 has unique functions that cannot be substituted by CAPN1 (Dutt *et al,* 2006). CAPNS1 knockout mice were also embryonically lethal and died at day 11.5. They show defects in cardiovascular system and erythropoiesis (Arthur *et al*, 2000). In contrast, CAPNS1 heterozygous mice were phenotypically normal. Mouse embryonic fibroblasts (MEFs) lacking CAPNS1 totally diminished calpain 1 and calpain 2 activities revealing the importance of CAPNS1 in calpain activation and embryonic development (Tan *et al*, 2006b).

CHAPTER II

A CALCIUM-DEPENDENT PROTEASE AS A POTENTIAL THERAPEUTIC TARGET FOR WOLFRAM SYNDROME

Summary

Wolfram syndrome is a genetic disorder characterized by diabetes and neurodegeneration and considered an endoplasmic reticulum (ER) disease. Despite the underlying importance of ER dysfunction in Wolfram syndrome and the identification of two causative genes, *WFS1* and *WFS2*, a molecular mechanism linking the ER to death of neurons and β cells has not been elucidated. Here we show that calpain is a therapeutic target for Wolfram syndrome. Calpain 2 is negatively regulated by WFS2, and elevated activation of calpain 2 by WFS2-knockdown correlates with cell death. Calpain activation is also induced by high cytosolic calcium mediated by the loss of function of WFS1. Calpain hyper-activation is observed in the WFS1 knockout mouse as well as in neural progenitor cells derived from induced pluripotent stem (iPS) cells of Wolfram syndrome patients. A small-scale small-molecule screen targeting ER calcium homeostasis reveals that dantrolene can prevent cell death in neural progenitor cells derived from Wolfram syndrome iPS cells. Our results demonstrate that the pathway leading to calpain activation provides potential therapeutic targets for Wolfram syndrome and other ER diseases.

Introduction

The endoplasmic reticulum (ER) takes center stage for protein production, redox regulation, calcium homeostasis, and cell death (Ron & Walter, 2007; Tabas & Ron, 2011). Thus, it is little wonder that its genetic or acquired dysfunction can trigger a variety of common diseases, including neurodegenerative diseases, metabolic disorders, and inflammatory bowel disease (Hetz *et al*, 2013; Wang & Kaufman, 2012). Breakdown in ER function can also lead to genetic disorders such as Wolfram syndrome characterized by diabetes, optic atrophy, diabetes insipidus, and neurodegeneration (Fonseca *et al*, 2005, 2010; Barrett *et al*, 1995). It is challenging to determine the exact effects of ER dysfunction on the fate of affected cells in common diseases with polygenic and multifactorial etiologies. In contrast, we reasoned that it should be possible to define the role of ER dysfunction in mechanistically homogenous patient populations, especially in rare diseases with a genetic basis, such as Wolfram syndrome (Urano, 2014).

Wolfram syndrome (OMIM #222300) is a rare autosomal recessive disorder characterized by juvenile-onset diabetes mellitus and bilateral optic atrophy (Barrett *et al*, 1995). Insulin dependent diabetes usually occurs as the initial manifestation during the first decade of life, while the diagnosis of Wolfram syndrome is invariably later with onset of the other features in the second and ensuing decades (Barrett *et al*, 1995; Marshall *et al*, 2013; Hershey *et al*, 2012). Two causative genes for this genetic disorder have been identified and named *WFS1* and *WFS2* (Inoue *et al*, 1998; Amr *et al*, 2007). It has been shown that multiple mutations in the WFS1 gene, as well as a specific mutation in the WFS2 gene, lead to β cell death and neurodegeneration through ER and

mitochondrial dysfunction (Fonseca *et al*, 2005, 2010; Chen *et al*, 2009b; Wiley *et al*, 2013; Shang *et al*, 2014). WFS1 gene variants are also associated with a risk of type 2 diabetes (Sandhu *et al*, 2007). Moreover, a specific WFS1 variant can cause autosomal dominant diabetes (Bonnycastle *et al*, 2013), raising the possibility that this rare disorder is relevant to common molecular mechanisms altered in diabetes and other human chronic diseases in which ER dysfunction is involved.

Despite the underlying importance of ER malfunction in Wolfram syndrome and the identification of WFS1 and WFS2 genes, a molecular mechanism linking the ER to death of neurons and β cells has not been elucidated. Here we show that the calpain protease provides a link between the ER and death of neurons and β cells in Wolfram syndrome.

Results

The causative gene for Wolfram syndrome, WFS1, encodes a ubiquitously expressed protein and has especially high expression in pancreatic β cells and neurons (Amr *et al,* 2007; Inoue *et al,* 1998; Fonseca *et al,* 2005). The localization of WFS2 is still controversial. To study the tissue and cellular localization of WFS2, we did immunoblot and immunofluorescence staining of various tissues and cells. We found that WFS2 is a ubiquitously expressed protein with high expression in the brain tissue (Figure 2.1 A). WFS2 protein is not highly expressed in the pancreas, but mainly exists in islet cells (Figure 2.1 B). Similar to WFS1, WFS2 protein is also localized to the ER (Figure 2.1 C). Mutations in the WFS1 or WFS2 have been shown to reduce neuronal and β cell mass in patients. But there was no animal model to study the disease with respect to cell death. Existing WFS1 knockout mice include the WFS1 β cell specific knockout mice and WFS1 whole body knockout mice. WFS1 β cell specific knockout mice do not have diabetes or β cell loss probably due to the fact that mice have stronger β cells than humans. Wfs1 whole body knockout do not show significant neural phenotypes. Therefore, we made WFS1 brain specific knockout mice by crossing WFS1 floxed/floxed mice with Nestin- CRE mice. We also imported WFS2 whole body knockout mice, which have been well studied in the aspect of aging and longevity but not Wolfram syndrome. After careful examination of the brain specific knockout mice along with the WFS2 whole body knockout mice, we determined tissue loss in a very specific

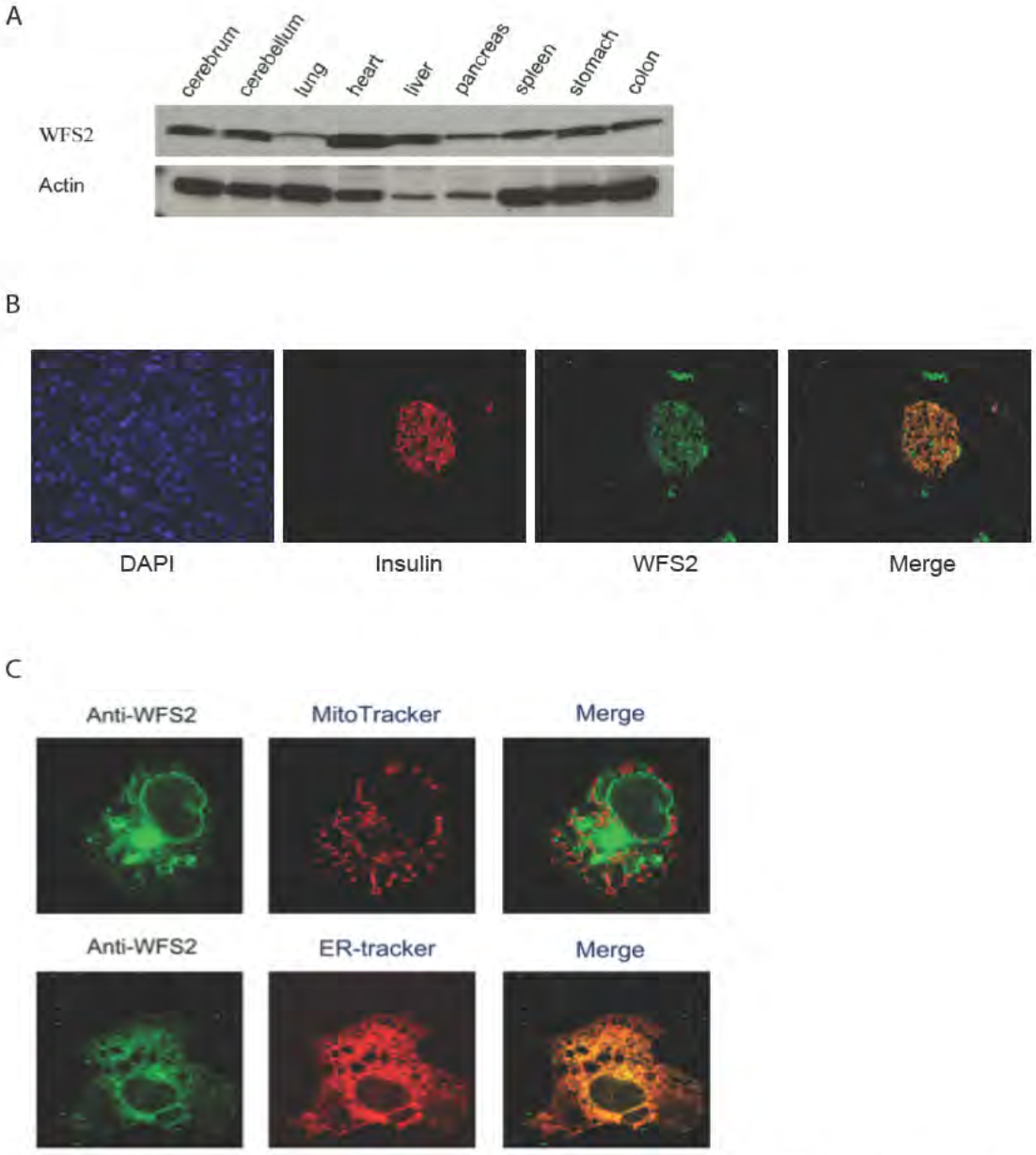


Figure 2.1 WFS2 expression in tissues and cellular localization. (A) Protein lysates were isolated from various mouse tissues. 50 μg of total protein was loaded for each lane. Levels of WFS2 and actin were monitored by immunoblotting. (B) Mouse pancreatic sections were fixed and stained for pancreatic islets and WFS2 by insulin and WFS2 antibodies. DAPI staining shows the cellular nucleus on the sections. Merged image of insulin and WFS2 staining is shown on the right panel. All images are shown under the same magnification. (C) Cos7 cells were co-stained with WFS2 and Mito-tracker (upper panels) or WFS2 and ER-tracker (lower panels). Merged images of WFS2 and Mito-tracker or WFS2 and ER-tracker staining are shown on the right panels.

region of the brain in both Wolfram syndrome mouse models (Figure 2.2). By comparing the brain slices with brain atlas, the region was identified as substantial nigra, which is also the pathologically effected region in Parkinson's disease. To confirm our observation, we stained the slides with tyrosine hydroxylase (TH), a dye for dopaminergic neurons, which are known to situate in the substantial nigra region of the brain, and saw perfect overlap of the tissue loss region and TH staining (Figure 2.3). This result implies that there is brain cell degeneration occurring.

To determine the cell death pathways emanating from the ER, we sought proteins that interact with Wolfram syndrome causative gene products. HEK293 cells were transfected with a GST-tagged WFS2 expression plasmid. The GST-WFS2 protein was purified along with associated proteins on a glutathione affinity resin. These proteins were separated by SDS-PAGE and visualized by Coomassie staining. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectroscopic analysis revealed 13 interacting proteins (Table 2.1), and one of the major WFS2 binding partners was CAPN2, the catalytic subunit of calpain 2, a member of the calcium dependent cysteine proteases family whose members mediate diverse biological functions including cell death (Goll *et al*, 2003; Tan *et al*, 2006a, 2006b) (Figure 2.4 A). Previous studies have shown that calpain 2 activation is regulated on the ER membrane and it plays a role in ER stress-induced apoptosis and β cell death (Tan *et al*, 2006a; Nakagawa & Yuan, 2000; Cui *et al*, 2013; Huang *et al*, 2010), which prompted us to study the role of WFS2 in calpain 2 activation.

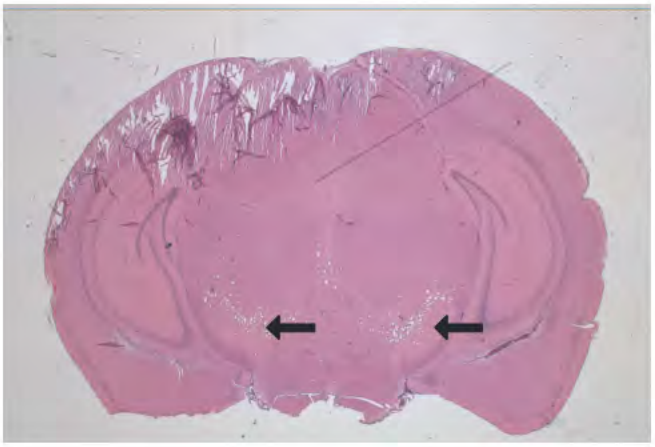


Figure 2.2 H&E staining of WFS1 knockout mice brain sections. WFS1 neuron specific knock out mice were generated by crossing WFS1 floxed mice with Nestin-Cre mice. Brain tissues isolated from these animals were fixed, sectioned, and stained hematoxylin and eosin. Back arrows show the symmetrical pattern of vesicles in the substantial nigra region of the brain.

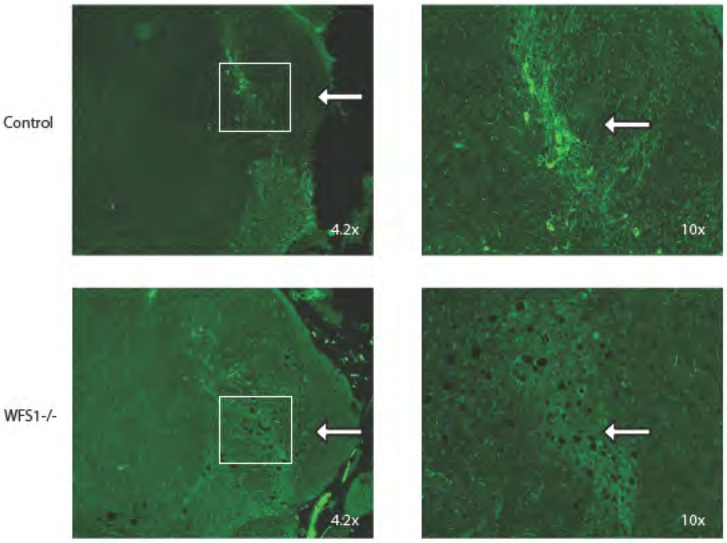


Figure 2.3 Loss of dopaminergic neurons in the substantia nigra region of WFS1 knockout mice brain sections. Immunofluorescence staining of brain sections from WFS1-/- (lower panels) and control littermate (upper panels) with tyrosine hydroxylase antibody, which specifically recognizes the dopaminergic neurons in the substantial nigra region. Left panels are 4.2x magnification. Right panels are zoomed image of the white boxed region. White arrows indicate the substantial nigra region of the brain section.

Calpain 2 is a heterodimer consisting the CAPN2 catalytic subunit and the CAPNS1 (previously known as CAPN4) regulatory subunit. We first verified that WFS2 interacts with calpain 2 by showing that endogenous calpain 2 subunits CAPN2 (Figure 2.4 B) and CAPNS1 (Figure 2.4 C) each associated with GST-tagged WFS2 expressed in HEK293 cells. Endogenous CAPN2 was also found to be co-immunoprecipated with N- or C-terminal FLAG-tagged WFS2 expressed in HEK293 cells (Figure 2.5 A and B respectively). To further confirm these findings, we performed a co-immunoprecipitation experiment in Neuro2a cells (a mouse neuroblastoma cell line) and INS-1 832/13 cells (a rat pancreatic β cell line) and found that endogenous WFS2 interacted with endogenous CAPN2 (Figure 2.4 D and E). WFS2 is known to be a transmembrane protein localized to the ER. We therefore explored the possibility that calpain 2 also localize to the ER. We transfected COS7 cells with pDsRed2-ER vector to visualize ER. Immunofluorescence staining of COS7 cells showed that endogenous calpain 2 was mainly localized to the cytosol, but also showed that a small portion co-localized with DsRed2-ER protein at the ER (Figure 2.4 F). Cell fractionation followed by immunoblot further confirmed this observation (Figure 2.5 C). Collectively, these results suggest that calpain 2 interacts with WFS2 at the cytosolic face of the ER.

Calpain hyper-activation has been shown to contribute to cell loss in various diseases (Goll *et al*, 2003), raising the possibility that calpain 2 might be involved in the regulation of cell death. To verify this issue, we overexpressed CAPN2, the catalytic subunit of calpain 2 and observed an increase of cleaved caspase-3 in HEK293 cells indicating that hyper-activation of calpain 2 induces cell death (Figure 2.4 G).

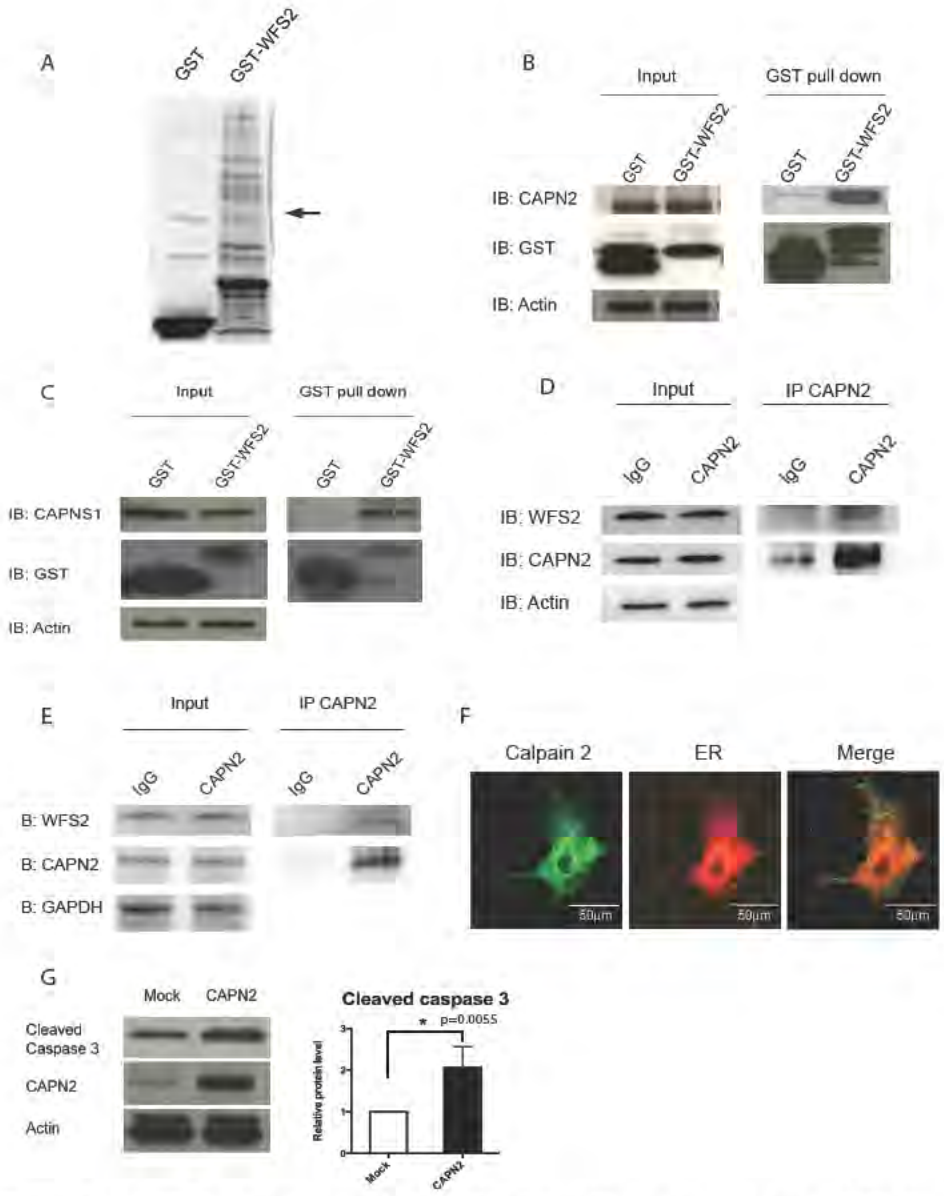


Figure 2.4 WFS2 interacts with CAPN2. (A) Affinity purification of WFS2-associated proteins from HEK293 cells transfected with GST or GST-WFS2 expression plasmid. Proteins were separated by SDS-PAGE and visualized by Coomassie blue staining. CAPN2 is denoted by an arrow. (B) GST-tagged WFS2 was pulled down on a glutathione affinity resin and the pulled-down products were analyzed for CAPN2. (C) GST-tagged WFS2 was pulled down on a glutathione affinity resin and the pulled-down products were analyzed for CAPNS1. (D) Lysates of Neuro2a cells were immunoprecipitated with IgG or anti-calpain 2 antibodies and analyzed for WFS2, CAPN2 or actin. (E) Lysates of INS-1 832/13 cells were immunoprecipitated with IgG or anti-calpain 2 antibody and analyzed for WFS2, CAPN2 or actin. (F) COS7 cells were transfected with pDsRed2-ER vector (middle panel) and stained with anti-calpain 2 antibody (left panel). (G) HEK293 cells were transfected with empty or a CAPN2 expression plasmid. Apoptosis was monitored by analysis of caspase 3 cleavage. Expression levels of CAPN2 and actin were measured by immunoblotting (left panel). Quantification is shown in right panel. (n=3, *P<0.05).

Order on gel	Gene Symbol	Full name	M.W.
1	PRKDC	DNA dependent protein kinase catalytic subunit	450 KDa
2	COPA	Coatomer Subunit alpha	140 KDa
3	IPO7, 4, 9	Importin 7, 4, and 9	120 KDa
4	XPO1, 2	Exportin 1, 2	110 KDa
5	MMS19	MMS19 nucleotide excision repair	110 KDa
6	CNX	Calnexinp	67 Kda
7	CAPN2	calpain-2	80KDa
8	GRP78	GRP78	78 KDa
9	TUBA TUBB	Alpha and Beta Tubulin	50 Kda

Table 2.1 GST-WFS2 interacting proteins

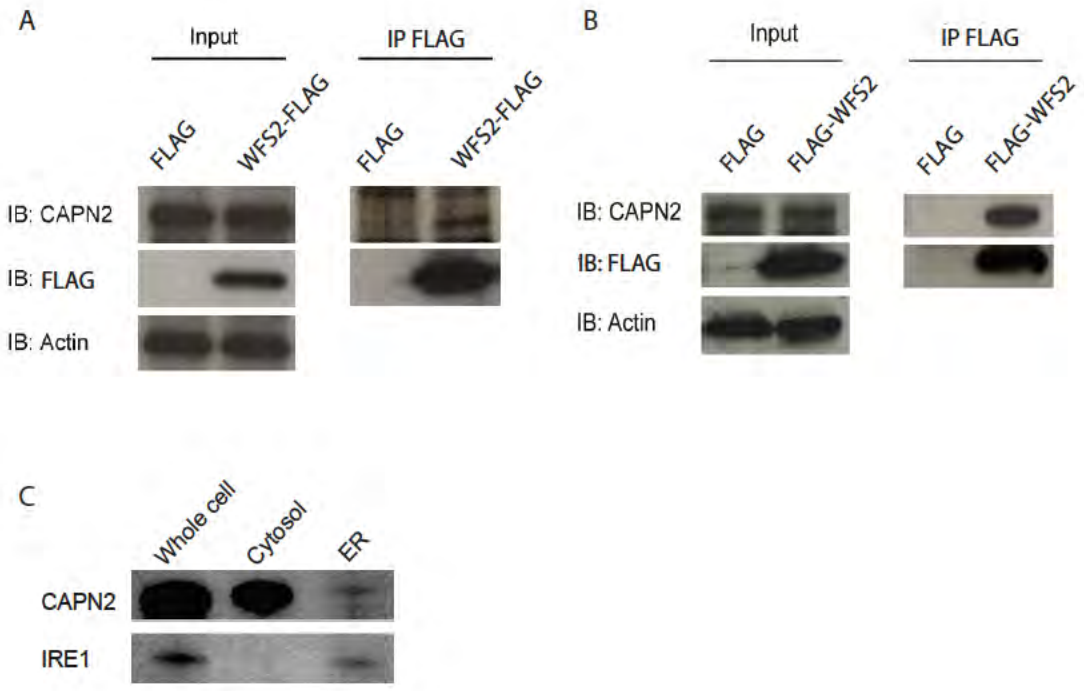


Figure 2.5 WFS2 and CAPN2 protein interacts. Cells lysates were immunoprecipitated from HEK293 cells transfected with empty FLAG vector or WFS2 expression plasmids with either a N-terminal (A) or a C-terminal FLAG-tag (B) and the immunoprecipitates were analyzed for CAPN2 by immunoblotting with anti-CAPN2 antibody. (C) Cells were fractionated into cytosolic and ER fractions. Localization of CAPN2 and IRE1 were determined by immunoblotting.

To determine whether WFS2 plays a role in cell survival, we suppressed WFS2 expression in mouse neuronal NSC34 cells using siRNA and measured cell death under normal and ER stress conditions. WFS2 knockdown was associated with increased cleavage of caspase 3 in normal or ER stressed conditions (Figure 2.6 A and 2B). We subsequently evaluated calpain 2 activation by measuring the cleavage of alpha II spectrin, a substrate for calpain 2. RNAi-mediated knockdown of WFS2 induced calpain activation, especially under ER stress conditions (Figure 2.6 A).

In patients with Wolfram syndrome, destruction of β cells leads to juvenile-onset diabetes (Barrett & Bundey, 1997). This prompted us to examine whether WFS2 was also involved in pancreatic β cell death. As was seen in neuronal cells, knockdown of WFS2 in rodent β cell lines INS1 832/13 (Figure 2.6 C) and MIN6 cells (Figure 2.8) was also associated with increased caspase-3 cleavage under both normal and ER stress conditions. The association of WFS2 with calpain 2 and their involvement in cell viability suggested that calpain 2 activation might be the cause of cell death in WFS2-deficient cells. To further explore the relationship between WFS2 and calpain 2, we expressed WFS2 together with the calpain 2 catalytic subunit CAPN2 and measured apoptosis. Ectopic expression of WFS2 significantly suppressed calpain 2-associated apoptosis under normal and ER stress conditions (Figure 2.7 A, lane 4 and lane 8, and Figure 2.7 B). Next, we tested whether CAPN2 mediates cell death induced by WFS2 deficiency. When CAPN2 was silenced in WFS2-deficient cells, apoptosis was partially suppressed compared with untreated WFS2-deficient cells (Figure 2.7 C). Taken together,

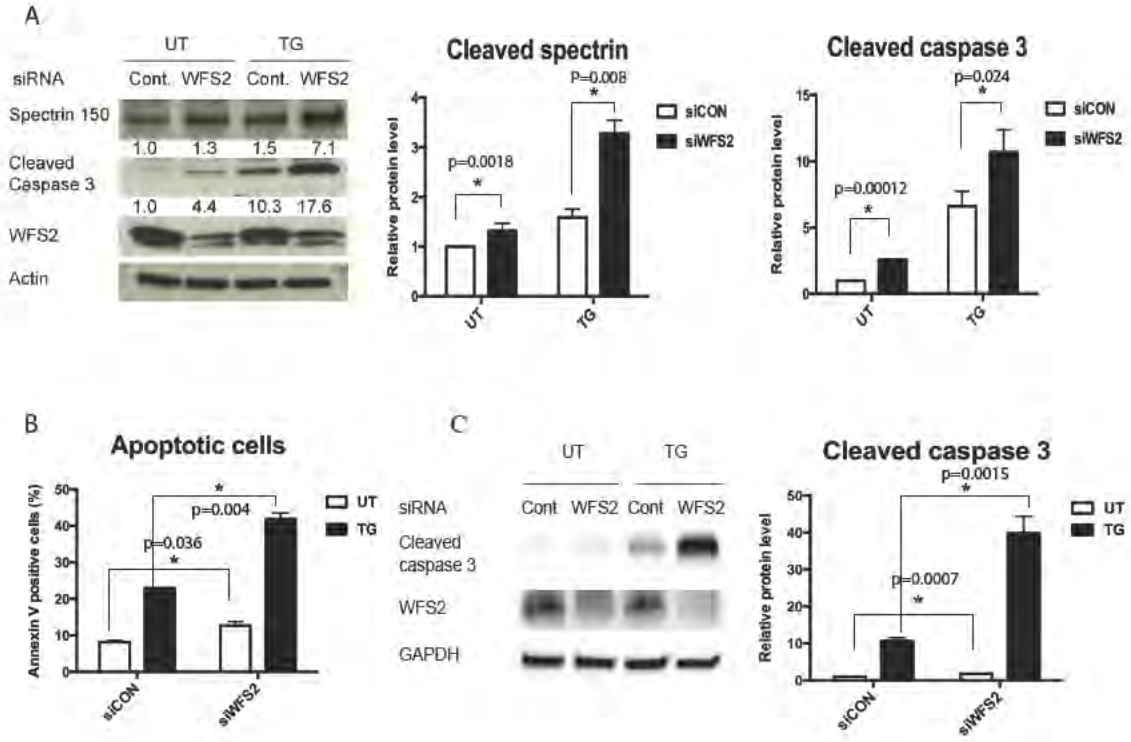


Figure 2.6 WFS2 deficiency induces cell death (A) NSC34 cells were transfected with control scramble siRNA or siRNA directed against WFS2, and then treated with 0.5μM thapsigargin (TG) for 6 h or untreated (UT). Apoptosis was monitored by immunoblotting analysis of cleaved caspase 3. Protein levels of cleaved spectrin, WFS2 and actin were measured by immunoblotting (left panel). Quantifications of cleaved spectrin and cleaved caspase 3 are shown in right panel, (n=5, *P<0.05). (B) NSC34 cells were transfected with control scramble siRNA or siRNA directed against WFS2, and then treated with 0.5μM thapsigargin (TG) for 6 h or untreated (UT). Apoptosis was monitored by Annexin V staining followed by flow cytometry analysis. (n=3, *P<0.05). (C) INS-1 832/13 cells were transfected with control scramble siRNA or siRNA directed against WFS2, and then treated with 0.5μM thapsigargin (TG) for 6 h or untreated (UT). Expression levels of cleaved caspase 3, WFS2 and actin were measured by immunoblotting (left panel). Protein levels of cleaved caspase 3 are quantified in right panel. (n=3, *P<0.05).

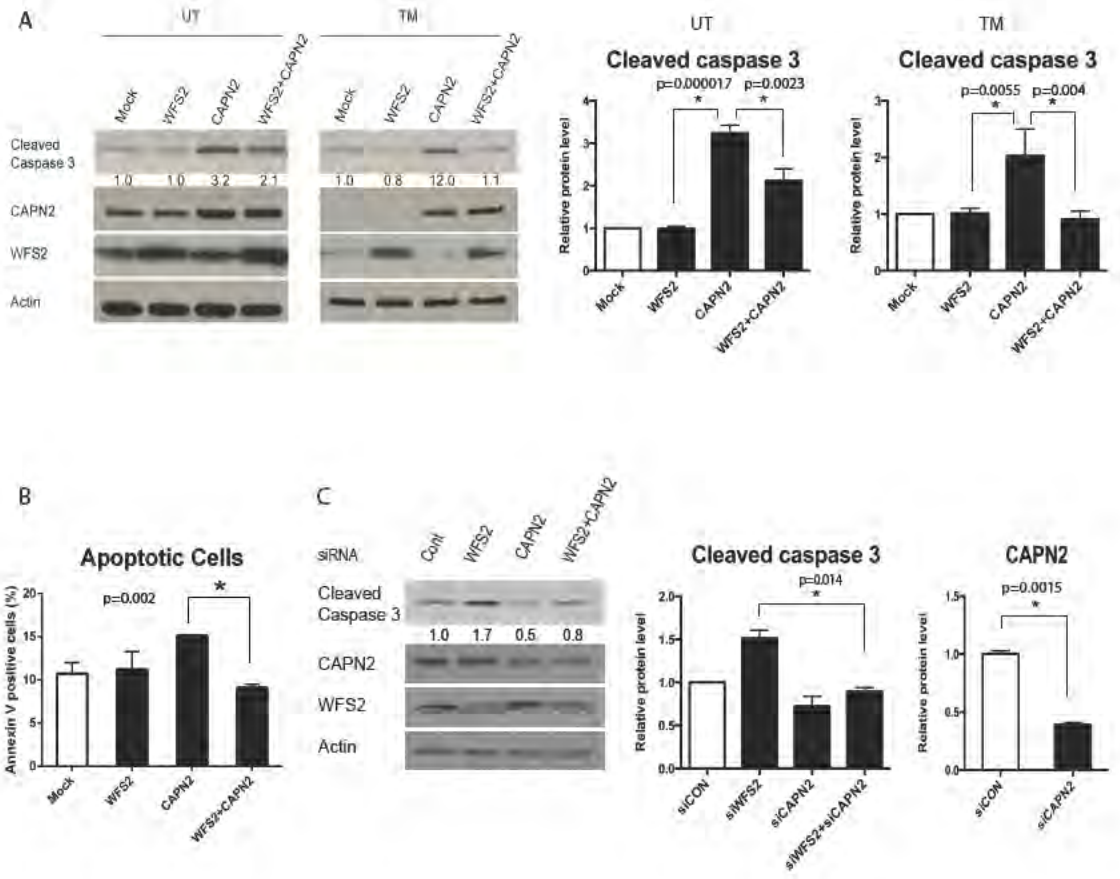


Figure 2.7 WFS2 suppresses cell death mediated by CAPN2. (A) NSC34 cells were transfected with empty expression plasmid (Mock), WFS2 expression plasmid, CAPN2 expression plasmid or co-tranfected with WFS1 and CAPN2 expression plasmids. Twenty-four h post transfection, cells were treated with 5μg/ml tunicamycin (TM) for 16 h or untreated (UT). Apoptosis was monitored by immunoblotting analysis of the relative levels of cleaved caspase 3 (indicated in left panel). Expression levels of CAPN2, WFS2, and actin were also measured by immunoblotting. Quantification of cleaved caspase 3 levels under untreated (middle panel) and tunicamycin treated (right panel) conditions are shown as bar graph. (n=5, *P<0.05). (B) Neuro2a cells transfected with empty expression plasmid (Mock), WFS2 expression plasmid, CAPN2 expression plasmid or co-transfected with WFS1 and CAPN2 expression plasmids were examined for apoptosis by Annexin V staining followed by flow cytometry analysis (right panel, n=3, *P<0.05). (C) NSC34 cells were transfected with scramble siRNA (Cont), WFS2 siRNA, CAPN2 siRNA or co-transfected with WFS2 siRNA and CAPN2 siRNA. Apoptosis was detected by immunoblotting of cleaved caspase 3. Protein levels of CAPN2, WFS2 and actin were also shown (left panel). Quantification of immunoblot is shown in right panel. (n=3, *P<0.05)

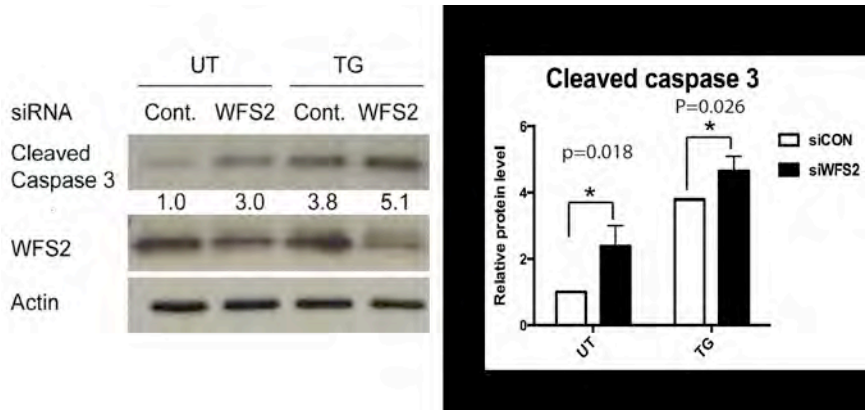


Figure 2.8 WFS2 deficiency induces apoptosis in MIN6 cells. MIN6 cells were transfected with control scramble siRNA or siRNA directed against WFS2, and then treated with 0.5 μM thapsigargin (TG) for 6 h or untreated (UT). Expression levels of cleaved caspase 3, WFS2 and actin were measured by immunoblotting. (left panel) Quantification of cleaved caspase 3 is indicated in right panel (n=3, *P<0.05).

these results suggest that WFS2 is a negative regulator of calpain 2 pro-apoptotic functions.

To further confirm that loss of function of WFS2 leads to cell death mediated by calpain 2, we tested if calpeptin, a calpain inhibitor, could prevent cell death in WFS2 deficient cells. In agreement with previous observations, calpeptin treatment prevented WFS2-knockdown-mediated cell death in neuronal (Figure 2.9 A and B) and β cell lines (Figure 2.9 C, and 2.11 A). Collectively, these results indicate that WFS2 is a suppressor of calpain 2-mediated cell death.

CAPN2 is the catalytic subunit of calpain 2. CAPN2 forms a heterodimer with the regulatory subunit, CAPNS1, which is required for protease activity and stability. We explored the role of WFS2 in CAPN2 and CAPNS1 protein stability. Ectopic expression or RNAi-mediated knockdown of WFS2 did not correlate with changes in the steady-state expression of CAPN2 (Figure 2.11 B). By contrast, overexpression of WFS2 significantly reduced CAPNS1 protein expression (Figure 2.10 A) and transient suppression of WFS2 slightly increased CAPNS1 protein expression (Figure 2.10 A). These data suggest that WFS2 might be involved in CAPNS1 protein turnover. Which is supported by the data showing GST-tagged WFS2 expressed in HEK293 cells associated with endogenous CAPNS1 (Figure 2.4 C). To investigate whether WFS2 regulates CAPNS1 stability through the ubiquitin-proteasome pathway, we treated HEK293 cells ectopically expressing WFS2 with a proteasome inhibitor, MG132, and then measured CAPNS1 protein level. MG132 treatment stabilized CAPNS1 protein in cells ectopically

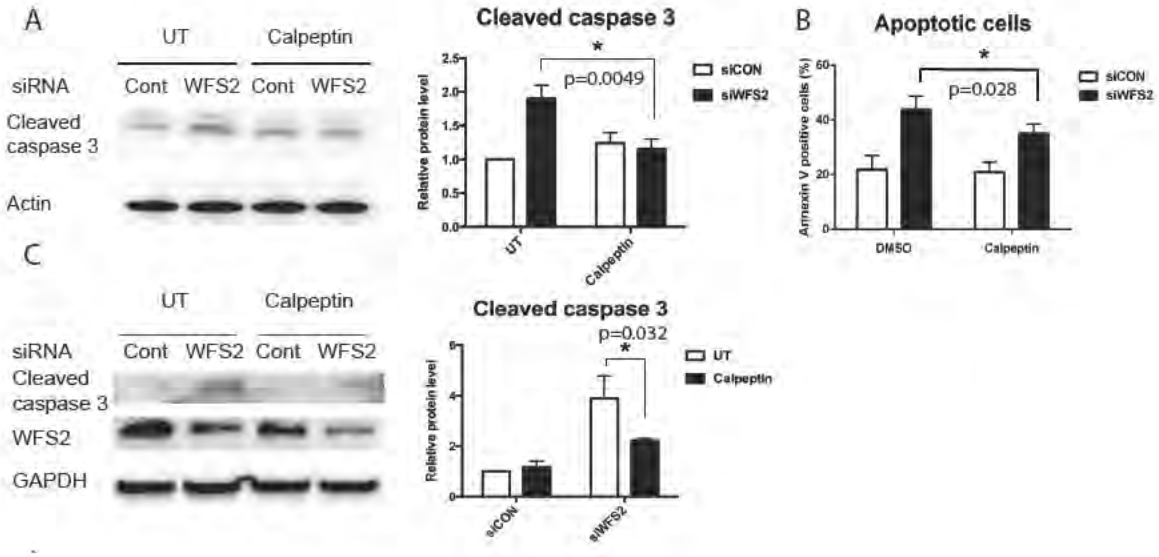
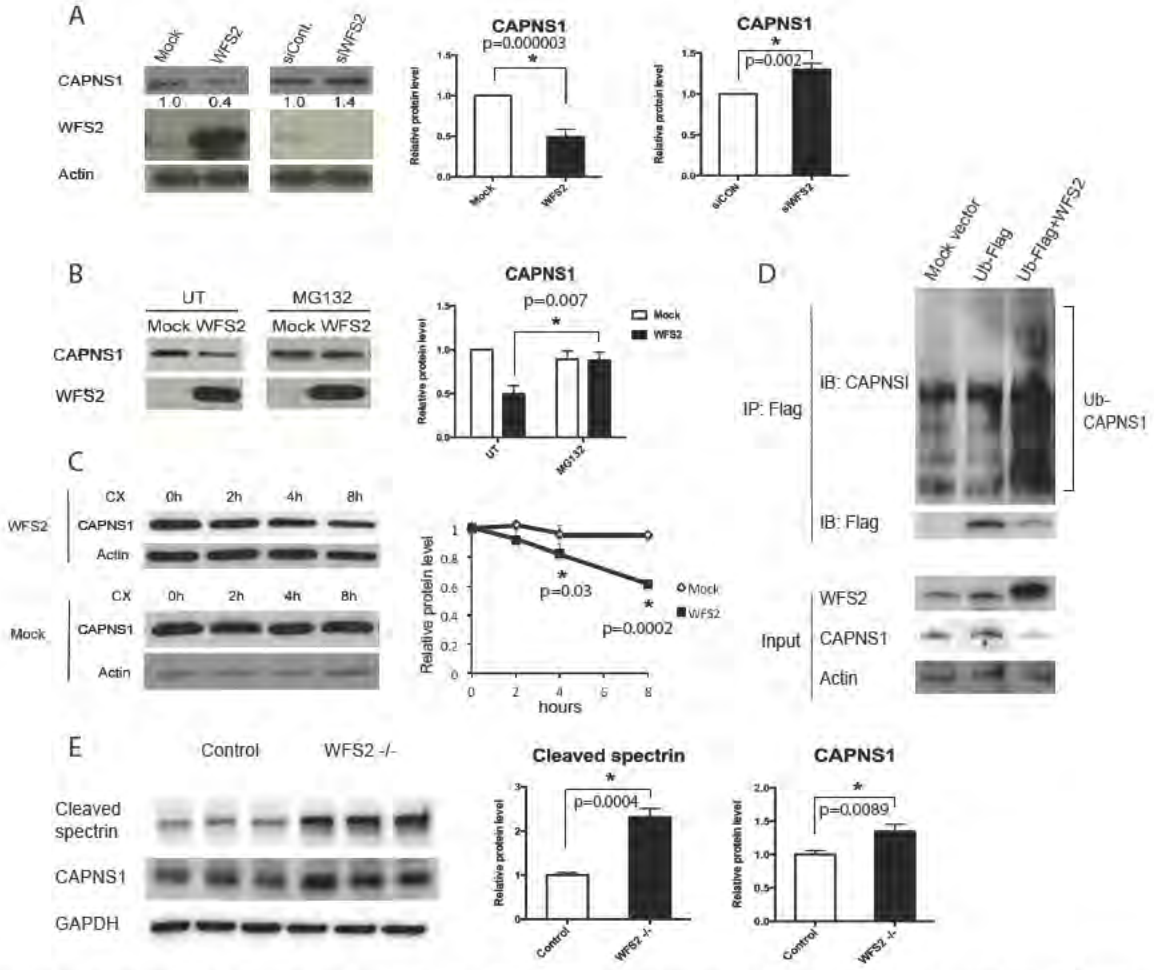


Figure 2.9 WFS2 regulates calpain activity. (A) Neuro-2a cells were transfected with siRNA against WFS2 and scramble siRNA. Thirty-six h after transfection, cells were treated with or without 100μM calpeptin for 12 h. Cleaved caspase 3 and actin levels were assessed by immunoblotting (left panel). Cleaved caspase 3 protein levels are quantified in right panel. (n=3, *P<0.05). (B) Neuro-2a cells were transfected with siRNA against WFS2 and scramble siRNA. Thirty-six h after transfection, cells were treated with or without 100μM calpeptin for 12 h. Early stage apoptosis are monitored by Annexin V staining followed by flow cytometry. (n=3, *P<0.05). (C) INS-1 832/13 cells were transfected with scramble siRNA and WFS2 siRNA. Twenty-four h after transfection, cells were treated with or without 5μM calpeptin for 24 h. Cleaved caspase 3, WFS2 and actin levels were monitored by immunoblotting (left panel) and quantified (right panel). (n=3, *P<0.05).



2.10 WFS2 regulates calpain activity though CAPNS1 (A) CAPNS1, WFS2, and actin levels were assessed by immunoblotting in HEK293 cells transfected with empty expression plasmid (Mock), WFS2 expression plasmid, scramble siRNA (siCON), or WFS2 siRNA (siWFS2) (left panel). Protein levels of CAPNS1 are quantified in right panels. (n=5, *P<0.05). (B) HEK293 cells were transfected with empty (Mock) or WFS2 expression plasmid, and then treated with MG132 (2 μM) or untreated (UT). Expression levels of CAPNS1 and WFS2 were measured (left panel) and quantified (right panel). (n=4, *P<0.05). (C) HEK293 cells were transfected with empty or WFS2 expression plasmid, and then treated with cycloheximide (100 μM) for indicated times. Expression levels of CAPNS1 and actin were measured (left panel). Band intensities corresponding to CAPNS1 in left panel were quantified by Image J and plotted as relative rates of the signals at 0 h (right panel). (n=3, *P<0.05). (D) NSC34 cells were transfected with mock empty vector, FLAG tagged ubiquitin (Ub-FLAG) plasmid or co-transfected with WFS2 expression plasmid and Ub-FLAG plasmid. Cell lysates were immunoprecipitated with FLAG affinity beads and analyzed for ubiquitin conjugated proteins. Levels of CAPNS1 and Ub-FLAG protein were measured in the precipitates. WFS2, CAPNS1 and actin expression was monitored in the input samples. (E) Brain lysates from control and WFS2 knockout mice were analyzed. Protein levels of cleaved spectrin and CAPNS1 were determined (left panel) and quantified (middle and right panel). (Each group n=3, *P<0.05).

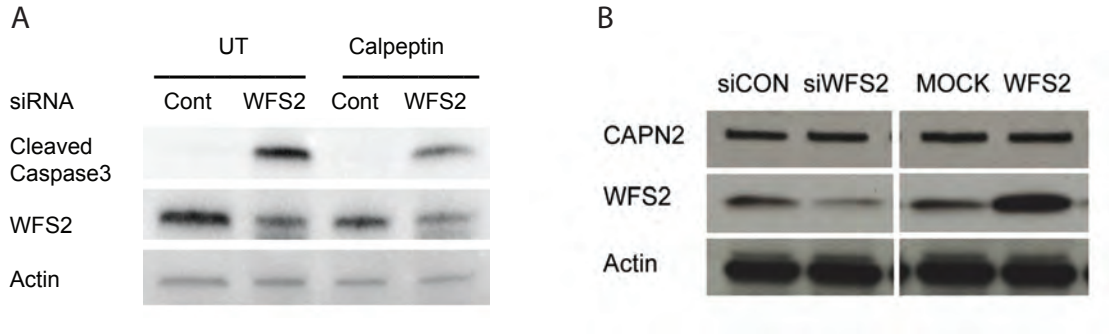


Figure 2.11 Calpeptin inhibits WFS2 knockdown induced apoptosis. (A) MIN6 cells were transfected with scramble siRNA and WFS2 siRNA. Thirty-six h after transfection, cells were treated with or without 100 μM calpeptin for 12 h. Cleaved caspase 3, WFS2 and actin levels were monitored by immunoblotting. (B) CAPN2, WFS2 and actin levels were assessed by immunoblotting in HEK293 cells transfected with scramble siRNA (siCON), WFS2 siRNA (siWFS2) (left), empty expression plasmid (Mock), or WFS2 expression plasmid (right).

expressing WFS2 (Figure 2.10 B). Furthermore, we performed cycloheximide chase experiments using HEK293 cells ectopically expressing WFS2 and quantified CAPNS1 protein level at different time points. Ectopic expression of WFS2 significantly decreased CAPNS1 protein levels at different time points. Ectopic expression of WFS2 was associated with significantly accelerated CAPNS1 protein loss, indicating that WFS2 contributes to post-translational regulation of CAPNS1 (Figure 2.10 C). To further assess whether WFS2 is involved in the ubiquitination of CAPNS1, we measured the levels of CAPNS1 ubiquitination in cells ectopically expressing WFS2 and observed that CAPNS1 ubiquitination level was increased by ectopic expression of WFS2 (Figure 2.10 D).

To further investigate the role of WFS2 in calpain 2 regulation, we collected brain lysates from WFS2 knockout mice and measured levels of cleaved spectrin, a well-characterized substate for calpain (Liu *et al*, 2006a). Notably, protein expression levels of cleaved spectrin as well as CAPNS1 were significantly increased in WFS2 knockout mice compared to control mice (Figure 2.10 E). Collectively, these results indicate that WFS2 inhibits calpain 2 activation by regulating CAPNS1 degradation mediated by the ubiquitin-proteasome system.

Calpain 2 is a calcium-dependent protease. WFS1, the other causative gene for Wolfram syndrome, has been shown to be involved in calcium homeostasis (Takei *et al*, 2006; Hara *et al*, 2014), suggesting that the loss of function of WFS1 may also cause calpain activation. To evaluate this possibility, we measured calpain activation levels in brain tissues from WFS1 brain specific knockout and control mice. We observed

significant increase of spectrin cleavage product, reflecting higher calpain activation levels in WFS1 knockout mice as compared to control mice (Figure 2.12 A). The suppression levels of WFS1 in different parts of the brain were shown in Figure 2.12 B. To further confirm that calpain is activated by the loss of WFS1, we looked for other calpain substrates in brain tissues from WFS1 knockout mice by a proteomics approach. Two-dimensional fluorescence difference gel electrophoresis experiment identified 12 proteins differentially expressed between cerebellums of WFS1 knockout mice and those of control mice (Figure 2.13 A and B). Among these, myelin basic protein (MBP) is a known substrate for calpain in the brain (Liu *et al*, 2006b). We measured myelin basic protein levels in brain lysates from WFS1 knockout and control mice. Indeed, the cleavage and degradation of myelin basic protein was increased in WFS1 knockout mice as compared to control mice (Figure 2.13 C).

Next, we looked for evidence of increased calpain activity in Wolfram syndrome patient cells. We created neural progenitor cells derived from induced pluripotent stem cells (iPSCs) of Wolfram syndrome patients with mutations in WFS1. Fibroblasts from four unaffected controls and five patients with Wolfram syndrome were transduced with four reprogramming genes (Sox2, Oct4, c-Myc, and Klf4) (Takahashi & Yamanaka, 2006) (Table 2.2). We produced at least 10 clones from each control- and Wolfram-iPSCs. All control- and Wolfram-iPSCs, exhibited characteristic human embryonic stem cell morphology, expressed pluripotency markers including ALP, NANOG, SOX2, SSEA4, TRA-1-81, and had a normal karyotype (Figure 2.14 A-F). To

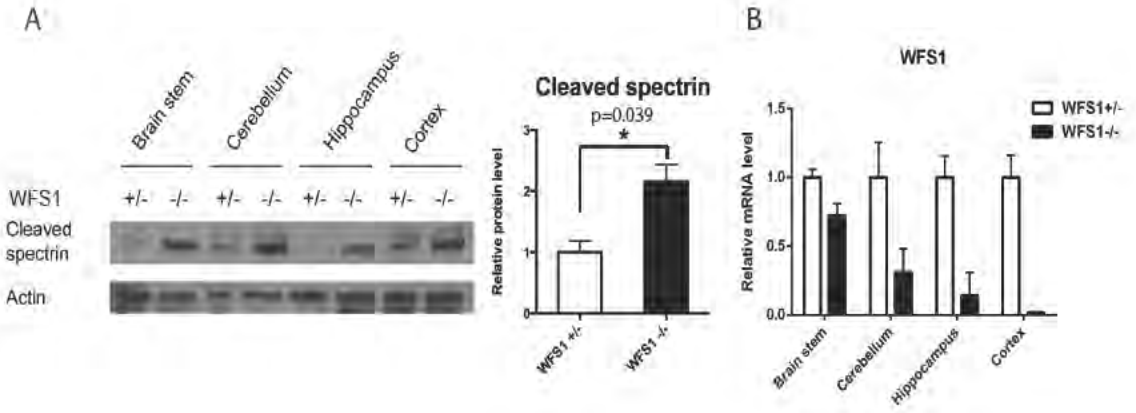


Figure 2.12 Evidence of Calpain 2 activation in a mouse model of Wolfram syndrome. (A) Protein was extracted from brain tissues of WFS1 brain specific knockout (-/-) and control (+/-) mice. Cleaved alpha II spectrin and actin levels were determined by immunoblot analysis (left panel). Quantification of cleaved spectrin is shown in right panel. (Each group n=10, *P<0.05). (B) WFS1 mRNA levels in different parts of brain in WFS1-/- and WFS1+/- mice were measured by qRT-PCR.

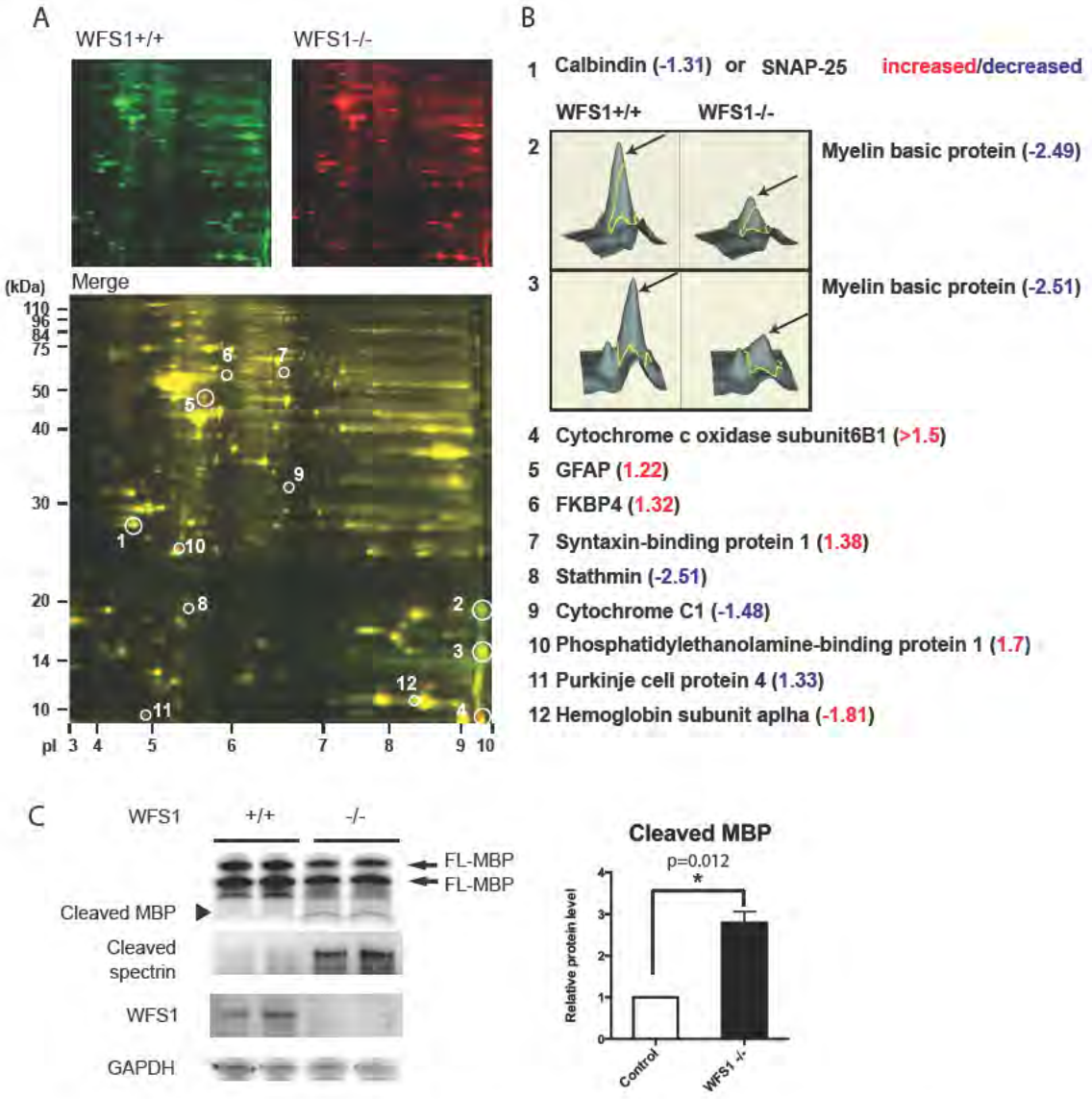


Figure 2.13 Induction of other calpain substrates in WFS1 animal model (A) 2-D fluorescence difference gel electrophoresis of cerebellum proteins from WFS1 knockout (WFS1-/-, labeled in red) and control (WFS1+/+, labeled in green) mice showing common (Merge, labeled in yellow) and unique proteins (circled). (B) The protein expression ratios between WFS1 knockout and control mice were generated, and differentially expressed spots were analyzed by MALDI-TOF mass spectrometry. Quantitative diagrams of spots #2 and #3, identified by mass spectrometry as myelin basic protein, showing lower levels of expression in WFS1 knockout mice compared with control mice. (C) Protein was extracted from cerebellums of WFS1 brain specific knockout (-/-) and control (+/+) mice. Cleaved myelin basic protein (black arrow), cleaved spectrin, WFS1 and GAPDH levels were determined by immunoblot analysis (left panel) and quantified in the right panel. (Each group n=3, *P<0.05).

iPSC line	Source	Clinical Diagnosis	WFS1 mutation	Sex	Age at biopsy	Age at onset of DM	Age at onset of OA	Deafness	DI
Wolf-2010-5	Washington University Wolfram Clinic	WFS	H313Y	F	15	3.8	12	1.7	NA
Wolf-2010-9	Washington University Wolfram Clinic	WFS	A126T; W613X	M	16	10.8	11	NA	14
Wolf-2010-11	Washington University Wolfram Clinic	WFS	A126T; W613X	M	10	7.5	6	8	10
Wolf-2010-13	Washington University Wolfram Clinic	WFS	L200fs286Stop; E752Stop	F	7	4.8	5.2	6	7.5
GM01610	Corriell Research Institute	WFS	W648X; G695V	F	11	NA	NA	NA	NA
BJ CRL-2522	ATCC	Control	NA	M	Newborn	NA	NA	NA	NA
Wolf-2010-5-MO	Washington University Wolfram Clinic	Control	None identified	F	41	NA	NA	No	No
Wolf-2010-9 MO	Washington University Wolfram Clinic	Control	NA	F	33	NA	NA	No	No
Wolf-2012-13-FA	Washington University Wolfram Clinic	Control	NA	M	42	NA	NA	No	No

WFS, Wolfram syndrome; DM, diabetes mellitus; OA, optic atrophy; DI, diabetes insipidus

Table 2.2 Information on genotypes and phenotypes of Wolfram syndrome and control subjects.

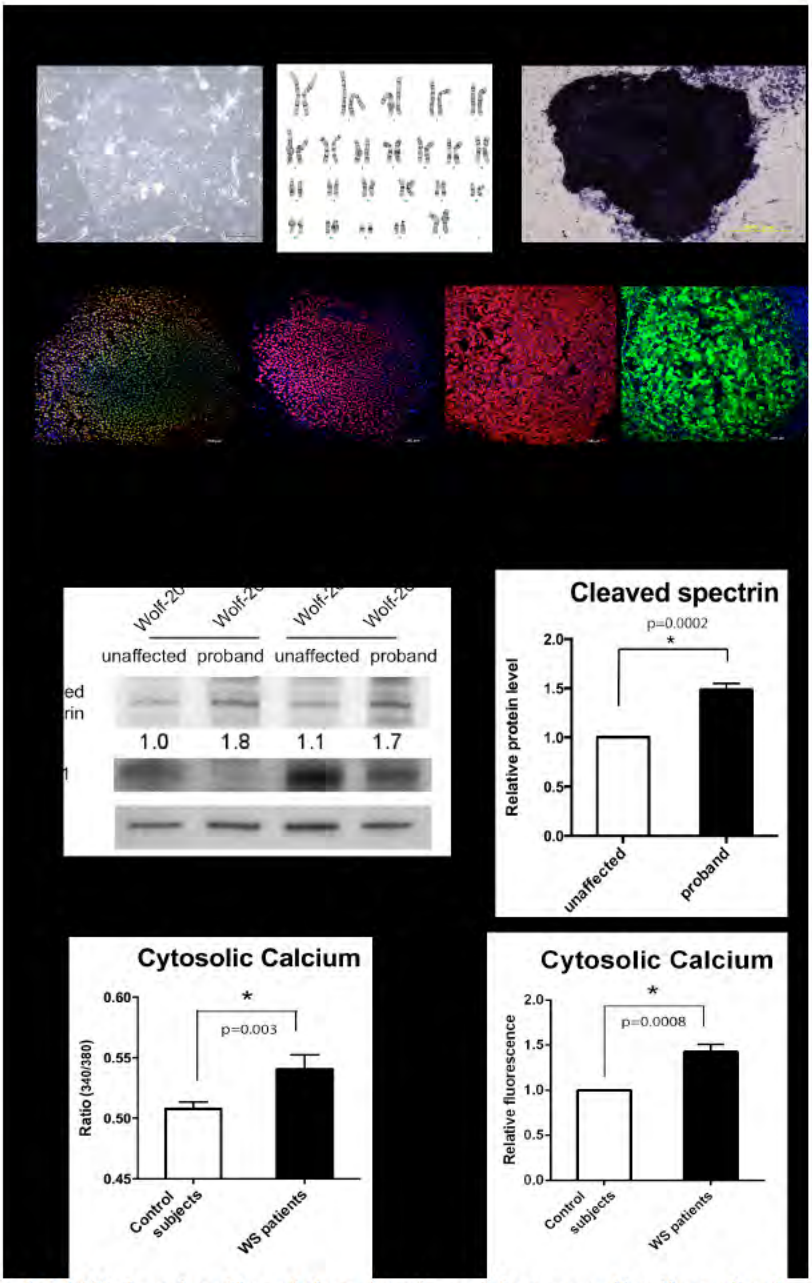


Figure 2.14 High cytosolic calcium levels and hyper-activation of calpain in patient neural progenitor cells. (A) Wolfram syndrome iPS cells derived from fibroblasts of a patient #1610 (left). Karyotype of the Wolfram iPS cells (right). (B) Alkaline phosphatase staining of the Wolfram iPS cells. (C-F) Wolfram syndrome iPS cells stained with pluripotent markers: Nanog (C), Sox2 (D), SSEA4 (D) and TRA-1 (F). (G) Immunoblot analysis of cleaved spectrin and actin in neural progenitor cells derived from Wolfram syndrome patient iPS cells. The relative levels of the spectrin cleavage product are indicated (left panel) and quantified (right panel) (n=4, *P<0.05). (H) (Left panel) quantitative analysis of cytosolic calcium levels in unaffected controls and Wolfram syndrome patients measured by Fura-2 calcium indicator. (All values are means ± s.e.m; n=6, * p<0.05). (Right panel) quantification of cytosolic calcium levels in unaffected controls and Wolfram syndrome patients measured by Fluo-4 calcium assay (n=4, * p<0.05).

create neural progenitor cells, we first formed neural aggregates from iPSCs. Neural aggregates were harvested at day 5, re-plated onto new plates to give rise to colonies containing neural rosette structures. At day 12, neural rosette clusters were collected, re-plated, and used as neural progenitor cells. In order to examine the differentiation potential of the neural progenitor cells, we tried to differentiate the neural progenitor cells into mature neurons. The differentiated neurons could be stained with TUJ1 antibody, indicating they are mature neurons. Interestingly, the control lines seem to differentiate more efficiently and form a better neural network then the Wolfram syndrome patient line. But this needs to be further investigated with more neural progenitor cell lines to draw any conclusions. Consistent with the data of WFS1 and WFS2 knockout mice, we observed that spectrin cleavage was increased in neural progenitor cells derived from Wolfram-iPSCs as compared to ones from control iPSCs, which indicates increased calpain activity (Figure 2.14 G).

Because calpain is known to be activated by high calcium, we explored the possibility that cytoplasmic calcium may be increased in patient cells by staining neural progenitor cells derived from control- and Wolfram-iPSCs with Fura-2, a fluorescent calcium indicator allowing accurate measurements of cytoplasmic calcium concentrations. Figure 2.14 H (left panel) shows that cytoplasmic calcium levels were higher in Wolfram-iPSCs derived cells than in control cells. This was confirmed by staining these cells with another fluorescent calcium indicator, Fluo-4 (Figure 2.14 H, right panel). Collectively, these results indicate that loss of function of WFS1 increases cytoplasmic calcium levels, leading to calpain activation. Next, we wanted to know whether there

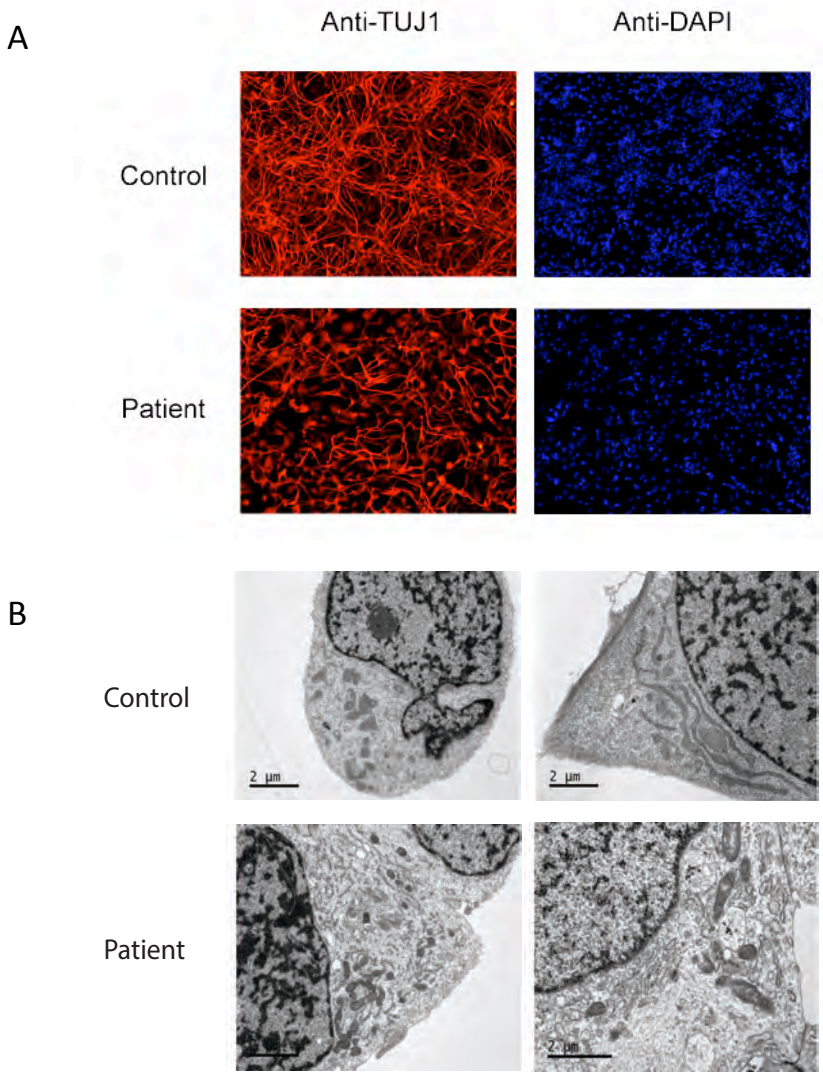


Figure 2.15 Differentiation and electron microscope scan of control and Wolfram syndrome patient neural progenitor cells (NPCs). (A) Differentiation of control (upper panels) and Wolfram syndrome patient (lower panels) NPCs. Neural progenitor cells were maintained in neural maturation media for 14 days. Then the cells were fixed and stained for mature neurons. Staining with anti-TUJ1 antibody is shown in red and DAPI staining of the neuron are shown in blue. (B) Electron microscopy scanning of control (upper panels) and patient (lower panels) NPCs.

were any visible damages in the organelles of the patient cells due to high calcium. We did electron microscope scanning of the neural progenitor cells from patients and control individuals, but did not detect any obvious damage in the organelles, which implies that the effect of increased cytosolic calcium is not acute. (Figure 2.15) This is consistent with the chronic pathological development history of Wolfram syndrome patients.

The results shown above demonstrate that the pathway leading to calpain activation provides potential therapeutic targets for Wolfram syndrome. To test this concept, we elected to focus on modulating cytosolic calcium and performed a small-scale screen to identify chemical compounds that could prevent INS-1 cell death mediated by calcium dysregulation caused by thapsigargin, a known inhibitor for ER calcium ATPase. Among 73 well-characterized chemical compounds that we tested (Table 2.3), 8 chemical compounds could significantly suppress thapsigargin-mediated cell death. These were PARP inhibitor, dantrolene, NS398, pioglitazone, calpain inhibitor III, docosahexaenoic acid (DHA), rapamycin, and GLP-1 (Figure 2.16 A). GLP-1, pioglitazone, and rapamycin are FDA-approved drugs, and have been shown to confer protection against ER stress-mediated cell death (Liu *et al*, 2006a; Yusta *et al*, 2006; Akiyama *et al*, 2009; Bachar-Wikstrom *et al*, 2013). Dantrolene is another FDA-approved drug clinically utilized for muscle spasticity and malignant hyperthermia (Michael & Dykes, 1975). It is of especially interesting because it had been shown to modulate ER and cytosolic calcium. Previous studies have shown that dantrolene is an inhibitor for the ER-localized ryanodine receptors and suppresses leakage of calcium

	Drugs	Treatment conc.
1	Nicotinamide(Vitamin B3)	10μM
2	Valproic acid	10μM
3	Sodium tauroursodeoxycholate(TUDCA)	10μM
4	(-)-Riboflavin(Vitamin B2,Vitamin G)	10μM
5	Thiamine hydrochloride(Vitamin B1 hydrochloride)	10μM
6	Memantine hydrochloride	10μM
7	(+-)-a-Lipoic acid	10μM
8	Kynurenic acid	10μM
9	Folic acid	10μM
10	Idebenone	10μM
11	Acetovanillone(Apocynin)	10μM
12	Aspirin	10μM
13	Pyridoxine hydrochloride	10μM
14	Dextromethorphan hydrobromide	10μM
15	2.3-Pyridinedicarboxylic acid(DPA)	10μM
16	R-(-)-Deprenyl hydrochloride(Selegiline hydrochloride)	10μM
17	NS-398	10μM
18	4-Aminobenzoic acid(PABA,Vitamin Bx, Vitamin H1)	10μM
19	Biotin	10μM
20	D-Pantothenic acid hemicalcium salt(Vitamin B5)	10μM
21	Chondroitin sulfate A sodium salt from bovine tracha(Glycosaminoglycans)	10μg/ml
22	Ebselen	10μM
23	PPBP maleate(4-PPBP maleate)	10μM
24	Minocycline hydrochloride	10μM
25	Pravastatin sodium salt hydrate	10μM
26	N-tert-Butyl-alpha-phenylnitrine(PBN)	10μM
27	Curcumin	10μM
28	TRO19622(Olesoxime)	10μM
29	Pyridoxamine dihydrochloride	10μM
30	Pyridoxal hydrochloride	10μM
31	Fibroblast Growth factor-Basic human	100ng/ml
32	Bryostatin1	100nM
33	Brain derived neurotrophic factor human	100ng/ml
34	SRP4988(PEDF)	100ng/ml
35	Erythropoietin	0.1UN/ml
36	Clioquinol	10μM
37	Kenpaullone	10μM
38	PARP inhibitor iii,DPQ	10μM
39	Glial Cell Line-derived Neurotrophic Factor human	100ng/ml
40	Ciliary Neurotrophic Factor, human	100ng/ml
41	Nitric Oxide Synthase, Neuronal Inhibitor 1(nNOS inhibitor)	10μM
42	Riluzole	10μM
43	Creatine	10μM
44	Anisomycin from streptomyces griseolus	10μM
45	NE 100 hydrochloride	10μM
46	Phenytoin	10μM
47	CsA	300nM
48	FK506	300nM
49	Rapamycin	10μM
50	Docosahexaenoic acid	10μM
51	GLP-1	50nM
52	Diazoxide	300μM
53	Glibenclamide	100μM
54	2-APB (2-Aminoethoxydiphenyl borate)	200nM
55	IL1RA	100ng/ml
56	Retinol	10μM
57	GW5015-16	10μM
58	GW9508	10μM
59	Etomoxir	20μM
60	Verapamil	20μM
61	Metformin	44uM
62	AICAR	10μM
63	pioglitazone	10μM
64	Troglitazone	10μM
65	N-Acetyl D-Shingosine	10μM
66	Dihydroceramide C2	10μM
67	Fumonisin B1	10μM
68	Ros inhibitor	100μM
69	SNAP	1mM
70	Dantrolene	10μM
71	Bcl XLBH4 human	1μM
72	Calp. Inhibitor iii	1μM
73	salburinal	25μM

Table 2.3 Chemical compounds used for a screen targeting ER calcium homeostasis.

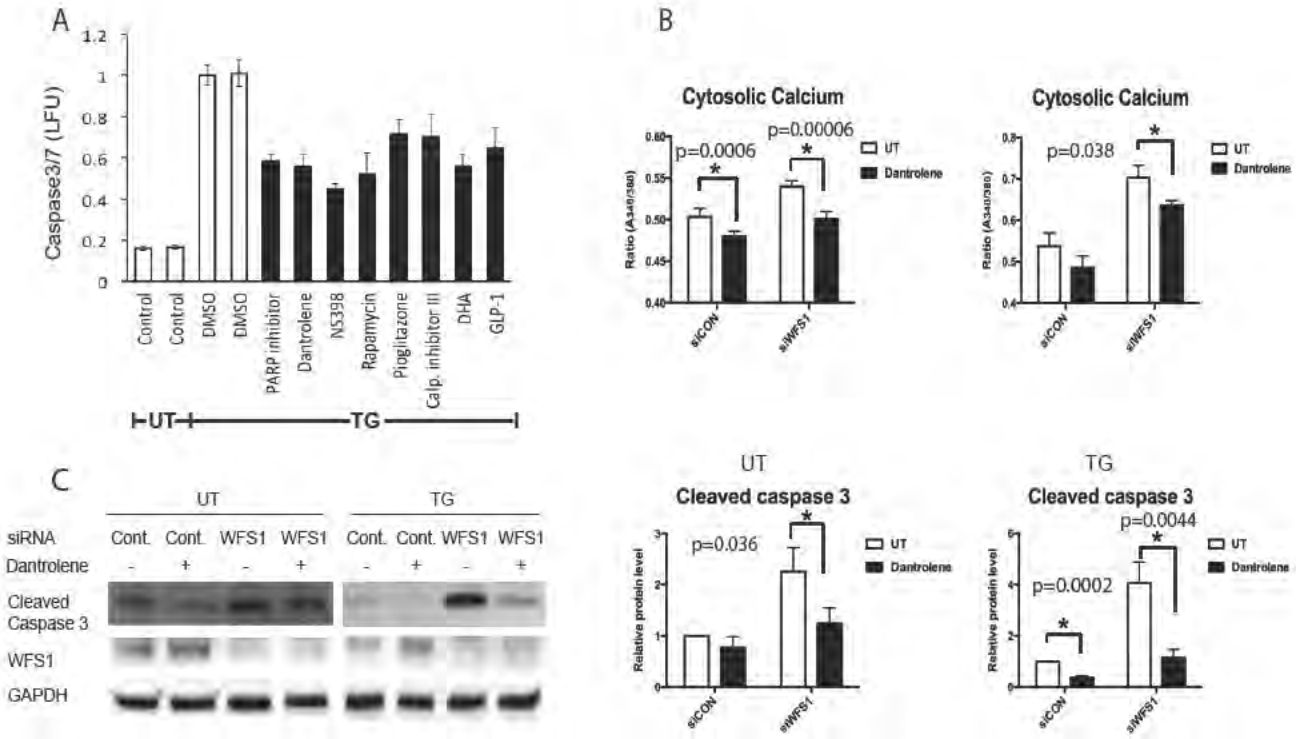


Figure 2.16 Dantrolene prevents cell death in Wolfram syndrome by inhibiting the ER calcium leakage to the cytosol. (A) INS-1 832/13 cells were pretreated with DMSO or drugs for 24 h then incubated in media containing 20 nM of thapsigargin (TG) overnight. Apoptosis was detected by caspase 3/7-Glo luminescence. (B) Cytosolic calcium levels were determined by Fura-2 in control and WFS1 deficient INS-1 832/13 (left panel) and NSC34 (right panel) cells treated or untreated with 10 μM dantrolene for 24 h. (All values are means ± s.e.m; n=6, * $p<0.05$). (C) INS-1 832/13 cells were transfected with scramble siRNA or siRNA against WFS1. Cells were pretreated with or without 10 μM dantrolene for 48 h, then incubated in media with or without 0.5 μM TG for 6 h. Expression levels of cleaved caspase-3, WFS1, GAPDH were measured by immunoblotting (left panel). Protein levels of caspase3 under untreated (middle panel) and TG treated (right panel) conditions are quantified and shown as bar graphs. (n=3, *$P<0.05$).

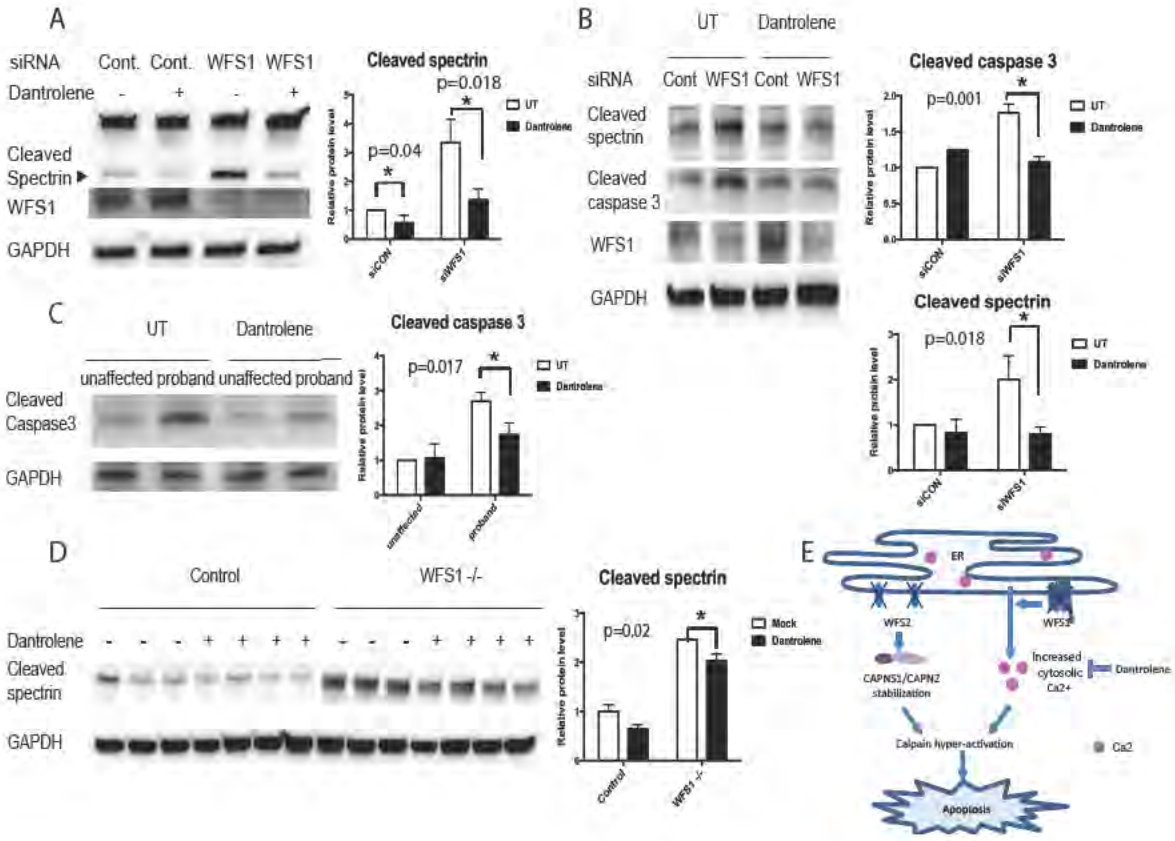


Figure 2.17 Dantrolene prevents cell death in iPS cell-derived neural progenitor cells and WFS1 mouse model (A) INS-1 832/13 cells were transfected with scramble siRNA or siRNA against WFS1, pretreated with or without 10 μM dantrolene for 48 h, then incubated in media containing 0.5 μM TG for 6 h. Protein levels of cleaved spectrin, WFS1, GAPDH were analyzed by immunoblotting (left panel) and quantified (right panel). (n=3, *P<0.05). (B) NSC34 cells were transfected with scramble siRNA or siRNA against WFS1. Then treated with or without10 μM dantrolene for 24 h. Protein levels of cleaved spectrin, cleaved caspase 3, WFS2 and GAPDH were determined by immunoblotting (left panel) and quantified (right panels) (n=3, *P<0.05). (C) Wolfram patient neural progenitor cells were pretreated with or without 10 μM dantrolene for 48 h. Then, cells were treated with 0.125 μM TG for 20 h. Apoptosis was monitored by immunoblotting (left panel). Quantification of cleaved caspase 3 protein levels are indicated in right panel. (n=3, *P<0.05). (D) Control and WFS1 brain specific knock out mice were treated with water or dantrolene for 4 weeks at 20 mg/kg. Brain lysates of these mice were examined by immunoblotting. Protein levels of cleaved spectrin and GAPDH were monitored (left panel) and quantified (right panel). (All values are means ± s.e.m; each group n>3, *P<0.05). (E) Scheme of the pathogenesis of Wolfram syndrome.

from ER to cytosol (Wei & Perry, 2002; Luciani *et al*, 2009). We thus hypothesized that dantrolene can confer protection against calcium dysregulation induced cell death in Wolfram syndrome, and performed a series of experiments to investigate this possibility. We first examined if dantrolene could decrease cytoplasmic calcium levels in WT cells. As expected, dantrolene treatment decreased cytosolic calcium levels in INS-1 832/13 and NSC34 cells (Figure 2.18 A and B). We next asked if dantrolene could restore cytosolic calcium levels in WFS1-deficient cells. RNAi-mediated WFS1 knockdown increased cytosolic calcium levels relative to control cells, and dantrolene treatment could restore cytosolic calcium levels in WFS1-knockdown INS-1 832/13 cells (Figure 2.16 B, left panel) as well as WFS1-knockdown NSC34 cells (Figure 2.16 B, right panel). Next, to determine whether dantrolene confers protection in WFS1 deficient cells, we treated WFS1 silenced INS-1 832/13 cells with dantrolene and observed suppression in apoptosis (Figure 2.16 C) and calpain activity (Figure 2.17 A). Dantrolene treatment also prevented calpain activation and cell death in WFS1-knockdown NSC34 cells (Figure 2.17 B). To verify these observations in patient cells, we pretreated neural progenitor cells derived from iPS cells of a Wolfram syndrome patient and an unaffected parent with dantrolene, and then challenged these cells with thapisgargin. Thapsigargin-induced cell death was increased in neural progenitor cells derived from the Wolfram syndrome patient as compared to those derived from the unaffected parent, and dantrolene could prevent cell death in the patient neural progenitor cells (Figure 2.17 C).

In addition, we also treated tissue specific WFS1 knockout mice with dantrolene and saw a protective effect. In brain-specific knockout mice, dantrolene could suppress

calpain hyper-activation (Figure 2.17 D), while in islet-specific knockout mice, dantrolene treated mice show slightly increased insulin content in islets and some improvement in glucose intolerance which was not significant due to the fact that the mice only developed mild glucose intolerance before the treatment, which give us a small dynamic range to work with (Figure 2.20 A-C). Collectively, these results argue that dantrolene could prevent cell death in Wolfram syndrome by suppressing calpain activation.

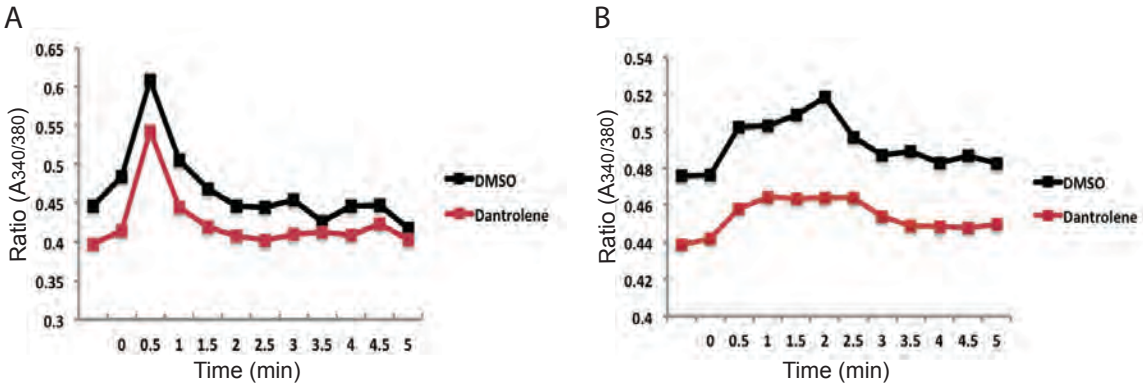


Figure 2.18 Reduction of cytosolic calcium levels with dantrolene treatment. INS-1 832/13 (A) and NSC34 (B) cells were pretreated with or without 10 μM dantrolene for 24 h. Cytoplasmic calcium levels were measured by Fura-2 calcium indicator over a period of time. Thapsigargin was added at 0 min time point (n=6).

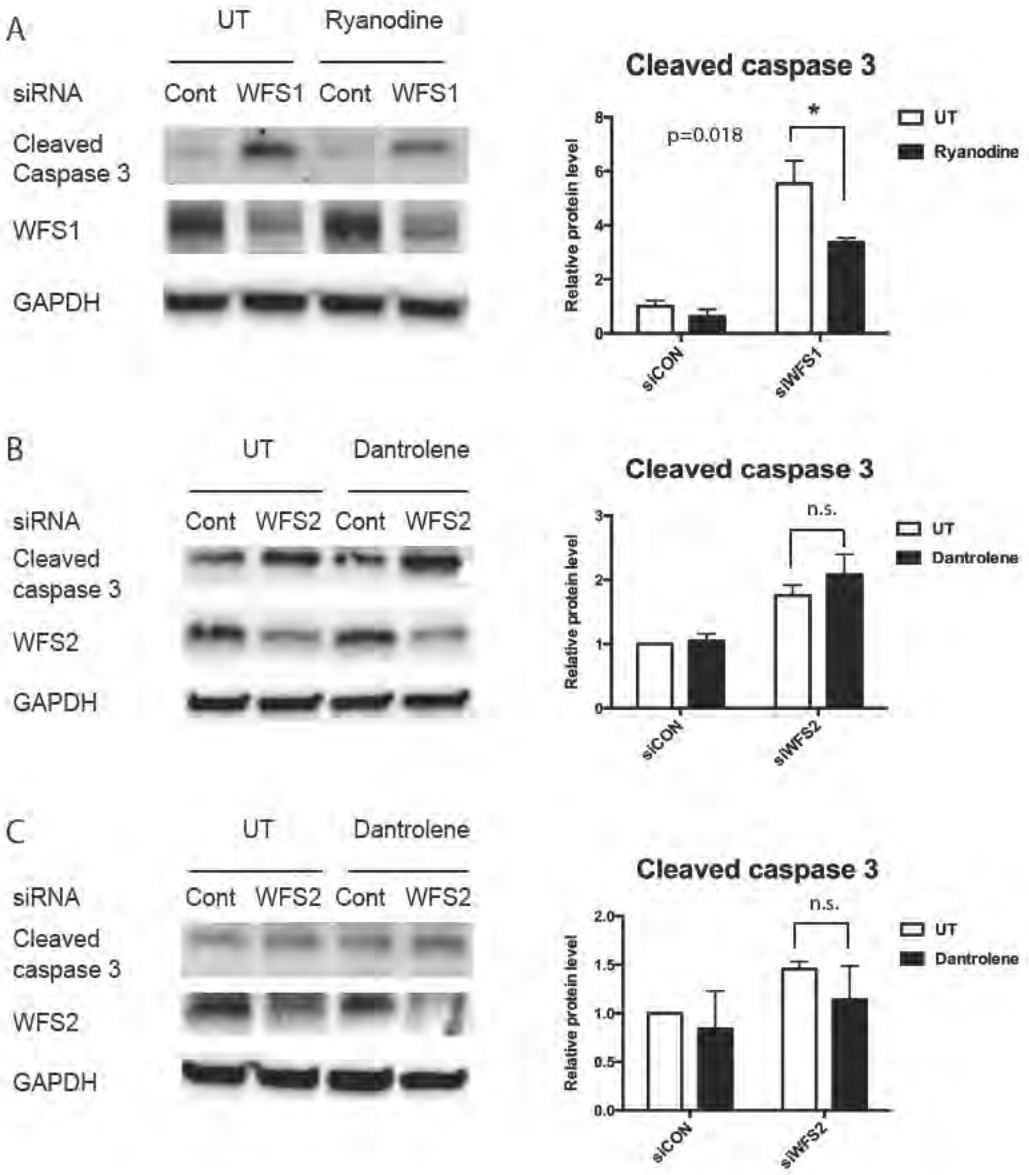


Figure 2.19 Apoptosis induced by high cytosolic calcium levels in WFS1 deficient cells can be treated with ryanodine (A) INS-1 832/13 cells were transfected with scramble siRNA and WFS1 siRNA. Twenty-four h after transfection, cells were treated with or without 2 μM ryanodine for 24 h. Cleaved caspase 3, WFS1 and GAPDH levels were monitored by immunoblotting (left panel) and quantified (right panel). (n=3, *P<0.05). (B) INS-1 832/13 cells and NSC34 cells (C) were transfected with scramble siRNA and WFS2 siRNA. Twenty-four h after transfection, cells were treated with or without 10 μM dantrolene for 24 h. Cleaved caspase 3, WFS2 and GAPDH levels were monitored by immunoblotting. Quantification of cleaved caspase 3 protein levels are shown in (D), (E) right panels respectively (n=3, *P<0.05).

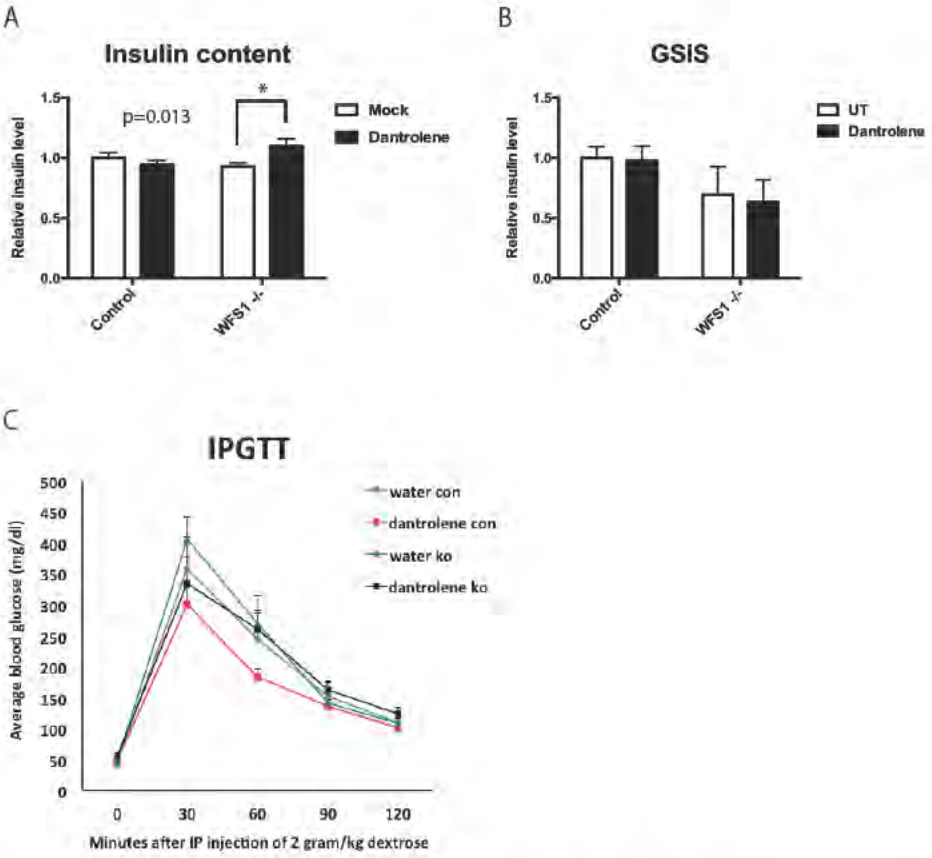


Figure 2.20 Islet function in control and WFS1 knockout mice. Control and WFS1 pancreatic β cell specific knock out mice were treated with water or dantrolene for 4 weeks at 20 mg/kg. Islets of these mice were isolated and examined for insulin content (A) and glucose stimulated insulin secretion (B). (C) Control and WFS1 pancreatic β cell specific knock out mice were treated with water or dantrolene for 4 weeks at 20 mg/kg. Intraperitoneal glucose tolerance test was performed at the end of the 4th week. (All values are means ± s.e.m; each group n=5, *P<0.05).

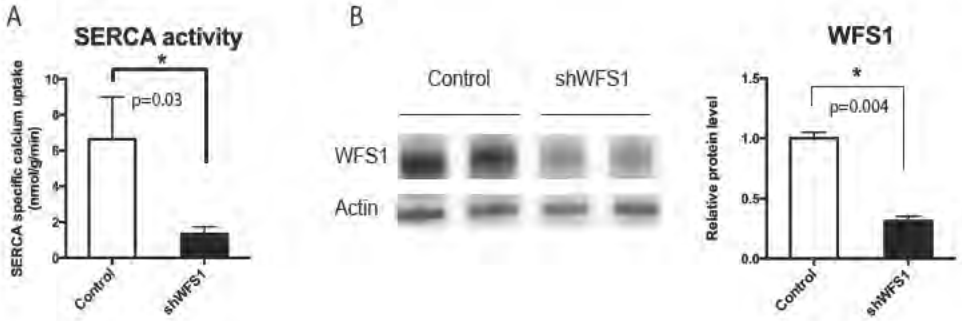


Figure 2.21 SERCA activity in WFS1 knockdown HEK293 cells (A) SERCA activity was measured in HEK293 cells stably expressing shRNA against scramble or WFS1 sequence. (All values are means ± s.e.m; n=6, *P<0.05). (B) Protein levels of WFS1 and actin were monitored in HEK293 cells stably expressing shRNA against scramble or WFS1 (left panel). Quantification is shown in right panel (n=3, *P<0.05).

Discussion

Growing evidence indicates that ER dysfunction triggers a range of human chronic diseases, including diabetes, atherosclerosis, inflammatory bowel disease, and neurodegenerative diseases (Hetz *et al*, 2013; Wang & Kaufman, 2012; Hotamisligil, 2010; Ozcan & Tabas, 2012). However, currently there is no effective therapy targeting the ER for such diseases due to the lack of clear understanding of the ER's contribution to the pathogenesis of such diseases. Although Wolfram syndrome is a rare disease and is currently neglected from mainstay drug discovery efforts, the homogeneity of the patient population and disease mechanism has enabled us to identify a potential target, a calcium-dependent protease, calpain. Our results provide new insights into how the pathways leading to calpain activation cause β cell death and neurodegeneration, which are schematically summarized in Figure 2.10 H.

There are two causative genes for Wolfram syndrome, WFS1 and WFS2. The functions of WFS1 have been extensively studied in pancreatic β cells. It has been shown that WFS1 deficient pancreatic β cells have high baseline ER stress levels and impaired insulin synthesis and secretion. Thus, WFS1-deficient β cells are susceptible to ER stress and cell death (Fonseca *et al*, 2005, 2010; Akiyama *et al*, 2009; Riggs *et al*, 2005; Ishihara *et al*, 2004; Zatyka *et al*, 2008). The functions of WFS2 are still not clear. There are evidences showing that impairment of WFS2 function can cause neural atrophy, muscular atrophy, mitochondrial dysfunction and accelerated aging in mice (Chen *et al*, 2009b). WFS2 has also been shown to be involved in autophagy (Chang *et al*, 2010). However, although patients with two genetic types of Wolfram syndrome suffer from the

same disease manifestations, it was not clear if a common molecular pathway was altered in these patients. Our study has demonstrated for the first time that calpain hyper-activation is the common molecular pathway altered in patients with Wolfram syndrome. The mechanisms of calpain hyper-activation are different in the two genetic types of Wolfram syndrome. WFS1 mutations cause calpain activation by increasing cytosolic calcium levels due to the dysfunction of ER calcium pump, whereas WFS2 mutations lead to calpain activation mainly due to impaired calpain inhibition.

Previously, Wolfram syndrome studies focused on pancreatic β cell function (Fonseca *et al*, 2005; Ishihara *et al*, 2004; Riggs *et al*, 2005). However, patients also suffer from neuronal manifestations. MRI scans of Wolfram syndrome patients showed atrophy in brain tissue implying neurodegeneration in patients (Barrett & Bundey, 1997; Hershey *et al*, 2012). In a mouse model of Wolfram syndrome, both WFS1 brain specific knockout mice and WFS2 whole body knockout mice showed sponging in the substantial nigra region of the midbrain. This sponging feature was previously seen in prion disease models, indicating loss of brain tissues. The sponging in Wolfram syndrome differs from prion disease in that it is restricted in a certain region while in prion disease, sponging occurs ubiquitously in the brain. Another difference we noticed is that the sponge holes in Wolfram syndrome seems larger then that of Prion's disease. The next step we should take in Wolfram syndrome study is to try to understand the function of substantial nigra, what specific cell types are there and which ones are affected. Substantial nigra is a structure located in the midbrain that functions in reward and movement. It appears in a darker color due to high population of dopaminergic neurons in the area. Previous studies

have show that a neurodegenerative disease, Parkinson's disease, patients experience loss of dopaminergic neurons in the substantial nigra region. The cause of death in dopaminergic neurons in Parkinson's disease is unknown. One theory is accumulation of misfolded proteins activates programmed cell death in these cells. Another theory is that dopaminergic neurons in Parkinson's patients contain less calbindin protein, leading to excess free calcium in the cytosol, which is toxic for cells. Interestingly, in Wolfram syndrome animal and cellular models, we also see induction of the UPR and high cytosolic calcium. This may hint that there are some common mechanisms between Parkinson's disease and Wolfram syndrome.

In order to investigate the mechanisms of neurodegeneration in Wolfram syndrome human cells, we established Wolfram syndrome iPSC-derived neural progenitor lines and confirmed the observations found in rodent cells and animal models of Wolfram syndrome, which is higher basal UPR levels and higher cytosolic calcium levels in Wolfram syndrome samples. Yet, further differentiation of the neural progenitor cells into specific types of neurons such as dopaminergic neurons should be carried out in the future to dissect out the cell types damaged in Wolfram syndrome. This is crucial for more thorough study of the disease and future drug treatments.

Calpain activation has been found to be associated with type 2 diabetes and various neuronal diseases including Alzheimers, traumatic brain injury and cerebral ischemia, suggesting that activation of calpains is crucial for cellular health (Cui *et al*, 2013). In our small-scale drug screening, we discovered that calpain inhibitor III could

confer protection against thapsigargin mediated cell death (Figure 6A). Our data also demonstrates that short-term calpeptin treatment was beneficial for cell with impaired WFS2 function. These results suggest that targeting calpain could be a novel therapeutic strategy for Wolfram syndrome. However, calpain is also an essential molecule for cell survival. Regulation of calpain activation level could be beneficial or toxic, thus we should carefully monitor calpain activity when treating patients with Wolfram syndrome (Dutt *et al*, 2006).

Calpain activation is tightly regulated by cytosolic calcium levels. In other syndromes that increase cytosolic calcium level in pancreatic β cells, patients experience a transient or permanent period of hyperinsulinaemic hypoglycemia. This can be partially restored by an inhibitor for ATP-sensitive potassium (KATP) channels or a calcium channel antagonist that prevents an increase in cytosolic calcium levels (Shah *et al*, 2014; Arya *et al*, 2014). Although patients with Wolfram syndrome do not experience a period of hyperinsulinaemic hypoglycemia, small molecule compounds capable of altering cellular calcium levels may hold promise for treating patients with Wolfram syndrome. Treatment of WFS1-knockdown cells with dantrolene and ryanodine could prevent cell death mediated by WFS1 knockdown. Dantrolene is a muscle relaxant drug prescribed for multiple sclerosis, cerebral palsy or malignant hyperthermia (Krause *et al,* 2004). Dantrolene inhibits the ryanodine receptors and reduces calcium leakage from the ER to cytosol, lowering cytosolic calcium level. First, we explored the effect of dantolene in vitro with neuronal and pancreatic β cell lines lacking WFS1, and found it to be protective. To further confirm the efficacy of dantrolene in vivo, we also treated WFS1

brain specific and WFS1 islet specific knockout mice with dantrolene for 4 weeks continuously. WFS1 brain specific knockout mice showed significant improvement in cleaved spectrin levels. While WFS1 islet specific knockout mice also showed some improvement in glucose intolerance, even though WFS1 islet specific knockout mice did not show significant impairment of islet function in the first place. This is probably due to the fact that mice have more robust β cells than humans. Dantrolene is widely used to treat muscle spasticity; it has also been studied in neurodegenerative diseases and proposed to be protective, but it has not been carefully studied in islets. This is the first time to show that it may be able to defend against diabetes related phenotypes in animal models. The protective effect of dantrolene treatment on WFS1 deficient cells and animals suggests that dysregulated cellular calcium homeostasis plays a role in the disease progression of Wolfram syndrome. In addition, it has been shown that stabilizing ER calcium channel function can prevent the progression of neurodegeneration in a mouse model of Alzheimer's disease (Chakroborty *et al*, 2012). Therefore, modulating calcium levels may be an effective way to treat Wolfram syndrome and even other ER diseases.

Dantrolene treatment did not block cell death mediated by WFS2 knockdown, suggesting that WFS2 does not directly affect the ER calcium homeostasis (Figure 2.19 B and C). RNAi-mediated WFS1 knockdown in HEK293 cells significantly reduced the activation levels of sarco/endoplasmic reticulum calcium transport ATPase (SERCA), indicating that WFS1 may play a role in the modulation of SERCA activation and ER calcium levels (Figure 2.21 A and B). It has been shown that WFS1 interacts with the

Na+/K+ATPase β1 subunit and the expression of WFS1 parallels that of Na+/K+ ATPase β1 subunit in a variety of settings, suggesting that WFS1 may function as an ion channel or regulator of existing channels (Zatyka *et al*, 2008). Further studies on this topic would be necessary to completely understand the etiology of Wolfram syndrome.

Our present study reveals that dantrolene can prevent ER stress-mediated cell death in human and rodent cell models as well as mouse models of Wolfram syndrome. Thus, dantrolene and other drugs that can regulate ER calcium homeostasis could be used to delay the progression of Wolfram syndrome and other diseases associated with ER dysfunction, including type 1 and type 2 diabetes.

Materials and Methods

Reagents

Thapsigargin, tunicamycin, calpeptin, dantrolene, ryanodine and cycloheximide were obtained from SIGMA (St. Louis, MO). RPMI-1640 and DMEM were from Invitrogen (Carlsbad, CA). Neural induction media, neural proliferation media were from Stemcell technologies (Vancouver, B.C., CA). MitoProbe DilC1(5) mitochondrial membrane potential assay kit, Annexin V Alexa Flour488 conjugate, Fluo-4 and Fura-2 calcium indicators were obtained from Invitrogen (Carlsbad, CA). Caspase-glo 3/7 protease assay kit and calpain-glo protease assay kit was purchased from Promega (Madison, WI). Mito Stress test kit was from Seahorse Bioscience (North Billerica, MA) Anti-WFS2 antibody, and anti-WFS1 antibody were purchased from Proteintech (Chicago, IL), anti-Caspase 3, anti-CAPN2 antibodies were obtained from Cell Signaling Technology (Danvers, MA), anti-CAPNS1 and anti-alpha II spectrin antibody were obtained from Millipore (Billerica, MA). Anti-actin antibody was purchased from SIGMA (St. Louis, MO). Anti-Myelin basic protein antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- Calpain 2 antibody, which detects both CAPN2 and CAPNS1, was raised in rabbits against bacterially expressed rat calpain 2.

Plasmids and siRNA

pCMV-SPORT6-WFS2 expression plasmid was purchased from Open Biosystems. pDsRed2-ER vector was purchased from Clonetech (Mountain View, CA). FLAG tagged WFS2 plasmids were constructed by inserting FLAG sequences into the N-

and C-termini of the expression plasmid. GST-WFS2 plasmid was generated by inserting WFS2 sequence into pEBG mammalian expression plasmid. A CAPN2 expression plasmid was generated in pLenti-CMV-puro plasmid provided by E. Campeau (Campeau et al., 2009). Lipofectamine2000 (Invitrogen, Carlsbad, CA) was used to transfect small interfering RNA (siRNA) directed against WFS2 and CAPN2 into cells. siRNAs were designed and synthesized at QIAGEN (Valencia, CA) as follows: mouse WFS2 CAACAGAAGGAUAGCUUG, human WFS2 CGAAAGUAGUGAAUGAAA, human CAPN2 CCGAGGAGGUUGAAAGUA. Rat WFS1 GUUUGACCGCUACAAGUUU. Cells were incubated in media overnight after siRNA transfection, and then additional treatments were performed, including ER stress induction.

Cell culture

Neuro-2a, NSC34, HEK293, MEFs and COS7 cells were cultured in DMEM containing 10% FBS, penicillin 100 U/ml and streptomycin 100 U/ml. MIN6 cells were grown in DMEM containing 15% FBS penicillin 100 U/ml and streptomycin 100 U/ml. INS-1 832/13 cells were cultured in RPMI containing 10% FBS, penicillin 100 U/ml, streptomycin 100 U/ml, 2-mercaptoethanol 3 μM, sodium pyruvate 1mM. Human fibroblasts were grown in FBM fibroblast basal medium supplemented with FGM-2 SingleQuots from Lonza (Basel, Switzerland), iPSCs were cultured in a feeder cell free system with mTeSR1 media from Stemcell Technologies (Vancouver, British Columbia, CA). Neural progenitor cells were generated in STEMdiff Neural Induction Media and maintained in STEMdiff Neural Progenitor Medium from Stemcell Technologies.

iPS cells and neural progenitor cells generation

To generate iPS cells, we obtained fibroblasts from non-affected controls and patients with Wolfram syndrome. Integration-free iPS cells were generated via Sendai viral delivery of the four reprogramming factors, Oct4, Sox2, Klf4, and c-Myc using Life Technologies' Cytotune reagents and protocol. All WFS- and control-iPSCs showed silencing of the four transgenes, exhibited characteristic human embryonic stem cell morphology, expressed pluripotency markers including ALP, NANOG, SOX2, SSEA4, TRA-1-81, and had a normal karyotype. To generate neural progenitor cells, iPSCs were counted and plated ~50,000 cells per well in a 96-well plate to form uniform embryoid bodies. After 5 days, embryoid bodies were suspended in neural induction media and replated as adherent cultures. Fresh media were applied every day for 7 days. Neural rosettes formed in these cultures were selected and plated. Plated rosettes were fed with STEMdiff Neural Induction Media every day for 4-7 days to obtain neural progenitor cells.

Maintenance of neural progenitor cells

Neural progenitor cells were plated on poly-ornithine (Sigma) and laminin (Sigma) double-coated plates (ready made poly-ornithine 37 ^{o}C, 2 h + laminin 10 μg/ml 37 ^{o}C, 2h). Plating density was ~1.0x10^{5} per cm^{2}. Cells were maintained in STEMdiff Neural Progenitor medium from Stemcell Technologies with daily medium changes until 100% confluent. Once the cells were confluent and ready for splitting, accutase was

applied to detach the cells. For freezing the cells, Bambanker cell freezing medium (Wako Chemicals, Osaka, JP) was used.

Differentiation of neural progenitor cells into mature neurons

Neural progenitor cells were cultured in maturation medium (neural basal medium A supplied with N2 supplement, B27 supplement, cAMP 10 μM, Noggin 500 ng/ml, retinoic acid 0.1 μM, SHH 50 ng/ml, BDNF 10 ng/ml, GDNF 10 ng/ml, IGF1 10 ng/ml) with daily medium changes for 2-3 weeks to obtain mature motor/ dopaminergic neurons.

MALDI-TOF mass spectrometry

HEK293 cells were transfected with GST-WFS2 plasmid and empty GST plasmid. Cell lysates were collected and immunoprecipitated with glutathione beads in lysis buffer (150 mM NaCl, 0.5% TritonX-100, 50 mM HEPES, 1 mM EDTA, 1 mM DTT, pH7.5). The precipitated proteins from both samples were resolved by SDS-PAGE and stained with Coomassie blue staining (Bio-Rad, Hercules, CA). The distinct bands that only appear in GST-WFS2 lane but not GST lane were analyzed by MALDI-TOF tandem mass spectrometry on a Shimadzu Axima TOF2 mass spectrometry at University of Massachusetts Medical School Proteomics and Mass Spectrometry Facility.

Immunostaining

Cells were fixed in 4% paraformaldehyde for 30 min at room temperature and then permeabilized with 0.1% Triton X-100 for 2 min. The fixed cells were washed twice

with PBS/Tween 0.1%, blocked with Image-It FX signal enhancer (Invitrogen, Carlsbad, CA) for 1 hour and incubated in primary antibody overnight at 4 °C. The next day, cells were washed three times in PBS/Tween 0.1% and incubated with secondary antibody for 1 h at room temperature, then cells were washed with PBS/Tween 0.1% for another three times and PBS twice and mounted with a Prolong Slowfade Reagents (Life Technologies, Carlsbad, CA) and sealed. Images were obtained with a Zeiss LSM 5 PASCAL confocal microscope with LSM Image software.

Hematoxylin and eosin (H&E) staining

Mouse brain specimen was fixed in 10% formalin for 2 days, embedded in paraffin, and processed for histopathologic examination. Paraffin-embedded serial sections were stained with hematoxylin and eosin (H&E).

Intraperitoneal glucose tolerance test (IPGTT)

Four weeks after dantrolene injection, WFS1 islet specific knockout mice (n=5 each group) were subjected to IPGTT. After 16 h fasting, glucose (2 g/kg) was injected intraperitoneally. Blood was drawn from end of the tail at time points 0, 30, 60, 90 and 120 min to measure blood glucose using a glucometer

Islet isolation

Pancreatic islets were isolated by pancreatic duct injection of 500 U/ml of collagenase solution followed by digestion at 37 °C for 40 minutes with mild shaking. Islets were then washed several times with Hank’s balanced salt solution (HBSS),

separated from acinar cells on a discontinuous Ficoll 400 gradient, viewed under a dissecting microscope and hand-selected.

Glucose stimulated insulin secretion (GSIS)

Thirty islets of similar size per mouse were picked after overnight culture and transferred into a 1.5ml tube. After incubation in 1ml of 3.3 mM glucose Kreb's (KRB) buffer (137 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, pH 7.4) for 1hr at 37°C, islets were then incubated in 1ml of 16.7mM glucose in KRB for another 2hr. Secreted insulin was quantified using supernatant of sample with insulin ELISA kit from Invitron (Monmouth, UK).

Insulin content

Thirty islets of similar size were picked per mice after overnight culture and lysed with T-PER tissue lysis buffer. Extracted protein was diluted and analyzed for insulin content by insulin ELISA kit from Invitron.

FACS analyses

For flow cytometry analyses, neural progenitor cells or NSC34 cells were plated in 24-well plates. After staining with the relevant fluorescence dye, cells were washed and resuspended in PBS. Flow cytometry analyses were performed with LSRII (Becton Dickson, Franklin Lakes, NJ) at the FACS core facility of Washington University School of Medicine. The results were analyzed by FlowJo ver.7.6.3.

Quantitative Real-Time PCR

Total RNA was extracted by RNeasy kits (Qiagen, Venlo, Limburg, NL). Reverse transcriptase PCR was performed using High Capacity Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) and quantitative PCR was demonstrated with Applied Biosystems ViiA7 using SYBR green dye.

2-D fluorescence difference gel electrophoresis

Proteins were extracted from cerebellums from WFS1 knockout mice and control mice. Equal amount of protein extract from paired samples were labeled by CyDye DIGE fluors, and the spectrally resolvable dyes enabled simultaneous co-separation and analysis of samples on a single multiplexed gel. These paired samples were simultaneously separated on a single 2D gel, using isoelectric focusing (IEF) in the first dimension and SDS polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. After electrophoresis, the gel was scanned using a Typhoon image scanner. Each scan revealed one of the CyDye signals (Cy3 and Cy5). ImageQuant software was used to generate the image presentation data including the single and overlay images. The comparative analysis of all spots was done by the DeCyder analysis software. The protein expression ratios between WFS1 knockout and control mice were generated, and differentially expressed spots were analyzed by MALDI-TOF mass spectrometry.

SERCA activity assay

The SERCA activity assay was performed as previously described in (Funai, Song et al. 2013). HEK293 cells were homogenized in hypotonic buffer, consisting of 10 mM $NaHCO_3$, 250 mM sucrose, 5 mM NaN_3, and 0.1 mM PMSF. ER fraction was

isolated using differential centrifugation. 125 to 300 mg of ER protein fraction were added to the assay mixture [100 mM KCL, 30 mM imidazole-histidine (pH 6.8), 5 mM $MgCl_2$, 5 mM ATP, 5 mM $(COOK)_2$, 5 mM NaN_3, and 50 μM $CaCl_2$ (10 uCi/μmol[^{45}Ca]; $CaCl_2$ American Radiolabeled Chemicals)] heated to 37^{o}C for 15 minutes. The reaction was stopped by the addition of 250 mM KCl and 1 mM $LaCl_3$. The mix was then vacuum filtered through a 0.2 μm HT Tuffryn membrane (Sigma). SERCA-dependent calcium transport was measured by comparing calcium transport with and without the presence of the 10 μM thapsigargin, a SERCA inhibitor.

Human Subjects

Wolfram syndrome patients were recruited through the Washington University Wolfram Syndrome International Registry website (http://wolframsyndrome.dom.wustl.edu/medical-research/Wolfram-Syndrome-Home.aspx). The clinic protocol was approved by the Washington University Human Research Protection Office and all subjects provided informed consent if adults and assent with consent by parents if minor children (IRB ID 201107067 and 201104010).

Animal experiments

WFS1 brain specific knockout mice were generated by breeding the Nestin-Cre transgenic mice (Jackson laboratory, Bar Harbor, ME) with WFS1 floxed mice (41). WFS2 whole body knock out mice are purchased from MRC Harwell (Oxfordshire, UK). All animal experiments were performed according to procedures approved by the

Institutional Animal Care and Use Committee at the Washington University in Saint Louis School of Medicine (A-3381-01).

Calcium levels

Calcium levels in cells were measured with an Inifinite M1000 plate reader (Tecan, Mannedorf, Switzerland). Neural progenitor cells or lymphocytes were plated in 96 well plates at a density of 25,000 cells per well and stained with 4 μg/ml Fura-2 (Invitrogen) dissolved in PBS for 30 min at 37 ^{o}C. 2.5 mM of probenecid (Invitrogen) was also added to the staining buffer to sustain the dye. Then the cells were washed with PBS supplemented with probenecid and incubated at room temperature for an additional 15 min. Fluorescence was measured at excitation wavelength 510 nm and emission wavelength 340 nm and 380 nm. Ratio of 340 nm and 380 nm was calculated.

Calcium levels in cells were measured by flow cytometry. Neural progenitor cells were plated in 12 well plate at a density of $5x10^5$ cells per well and stained with 4 μg/ml Fluo-4 dye dissolved in probenecid (2.5 mM) supplemented PBS for 30 min at 37 ^{o}C. Then cells were washed with probenecid containing PBS and incubated at room temperature for another 15 min. Fluorescence intensity of these cells was analyzed by flow cytometry.

Statistical analysis

Two-tailed t-tests were used to compare the two treatments. P values below 0.05 were considered significant. All values are shown as means ± S.D. if not stated.

CHAPTER III

PROSPECTIVES

Studying Wolfram syndrome is important for understanding diabetes and neurodegenerative diseases.

Globally as of 2013, an estimate of 381 million people had diabetes. This number is still increasing rapidly, especially in developing countries. By 2030, it is predicted that this number will double. In the United States, diabetes is the 7th leading cause of death. In 2012, an estimate of 29.1 million Americans (9.3% of the population) had diabetes and another 86 million adults were prediabetic. In 2013, diabetes cost $245 billion in United States and at least $500 billion globally (Yang *et al*, 2013).

Today, 35.6 million people worldwide suffer from Alzheimer's disease; 10 millions from Parkinson's; 2.5 million from multiple sclerosis (MS); 120,000 from amyotrophic lateral sclerosis (ALS), and over 350,000 from Huntington's disease. Because neurodegenerative diseases strike primarily later in life, the incidence is expected to soar as the population ages. As our average life span increase, 30 years from now, more than 12 million Americans will suffer from neurodegenerative diseases.

The challenge in understanding diabetes and neurodegenerative diseases is that they are complex disorders caused by multiple risk factors including genetics factors, lifestyles and environmental factors. Researchers have identified at least 20 chromosomal regions associated with type 1 diabetes, and more than a dozen genes linked to type 2

diabetes (Pociot & McDermott, 2002; Rich *et al*, 2008). Studies done on multiple sclerosis, a type of neurodegenerative disorder, revealed more than 19 regions on the genome are related to the disease (Sawcer *et al*, 1996). In addition to the large number of genes contributing to diabetes and neurodegenerative disorders, environmental factors and lifestyles such as diet and exercise also play a role that cannot be ignored. Discovering a risk factor and estimating its contribution to a complex disease is difficult, due to the fact that the contribution of each factor may be subtle or cofounded by other factors. What's more, the effect of environmental factors may obscure the impact of a single risk factor.

Studying a monogenetic disorder can provide insight into the contribution of a single gene in more complicated diseases. Although mutations are relatively rare, these genes are usually essential for health. Variants in monogenetic disorder genes have also been shown to be associates with common diseases (Brinkman *et al*, 2006). In the case of amyotrophic lateral sclerosis (ALS), there is a monogenetic form, which accounts for 2% of total ALS patients (Rosen, 1993). These patients have a gain of function mutation in the *SOD1* gene. By studying this monogenetic form of ALS, scientists were able to determine that mutant SOD1 induced damage of mitochondria, protease, and protein folding machinery were mechanisms contributing to the disease (Wong *et al*, 1995; Tobisawa *et al*, 2003; Nishitoh *et al*, 2008; Steinacker *et al*, 2014). Further studies proved that these mechanisms were also present in general ALS population (Swerdlow *et al*, 1998; Chattopadhyay & Valentine, 2009; Atkin *et al*, 2008; Ito *et al*, 2009). Thus, revealing the biological progression and molecular pathogenesis of monogenetic disorder

can contribute to discovery of novel therapeutic targets for both rare and common diseases.

Wolfram syndrome is an autosomal recessive monogenetic disorder characterized by juvenile-onset diabetes, optic atrophy and progressive neurodegeneration. Two causative genes for Wolfram syndrome have been identified—Wolfram syndrome 1 (*WFS1*) and Wolfram syndrome 2 (*WFS2*). Both prototypes of Wolfram syndrome are monogenetic disorders. WFS1 has also been shown to be a top risk factor in Type 2 diabetes. Understanding the disease mechanism for Wolfram syndrome may give us a clue of the pathogenesis of diabetes and neurodegenerative disorders. The goal of our study was to reveal the possible cause of cellular death in Wolfram syndrome and find a possible treatment. In this thesis, I showed that through calcium leakage or protein regulation, loss of WFS1 or WFS2 leads to hyperactivation of calpain, which latter induces apoptosis. This finding may shed light on mechanisms of more complex disorders that represent the same disease phenotypes. In the last part of chapter 2, I demonstrated that dantrolene might be a good mechanism-based drug for Wolfram syndrome. This may also apply to other diseases caused by disruption of calcium homeostasis.

Potential treatments for Wolfram syndrome and other genetic diseases

1. Genetic modification

For genetic diseases, especially monogenetic disease such as Wolfram syndrome, genetic medicine is a straightforward strategy. By transferring genetic

materials that are lacking in patients, we may be able to reverse the pathological phenotype. To date, genetic medicine approaches have been tested in clinic. The first attempt to cure human disease by gene transfer was in 1990, since then, 95 human trials have been carried out for gene transfer worldwide, all directed against monogenetic diseases (Blaese *et al*, 1995). These human trials proved that gene transfer could be applied in human patients to induce phenotypic modifications. But, gene transfer efficiency, host immune response, expression sufficiency, delivery specificity, possible mutagenesis are still challenges that we are facing. Further modification of the vectors and delivery methods are necessary before establishing it as a standard treatment for patients.

2. Chaperones and drugs

Previous studies showed that Wolfram syndrome is an ER stress disease. Loss of the WFS1 gene leads to continuous activation of UPR component ATF6, which further induce CHOP expression resulting in cell death. Eliminating ER stress in Wolfram syndrome might be beneficial for patients. Extensive studies on ER stressed showed that chaperones, either endogenous chaperones or chemical chaperones, are effective in reducing ER stress levels. Chaperones are small molecules that can facilitate and stabilize the folding of proteins. Endogenous protein chaperones in human includes BIP, which is a component of the UPR. Overexpression of BIP has been shown to be protective for many ER stress diseases. Chemical chaperones are small compounds whose functions include non-specifically stabilizing misfolded proteins, preventing protein aggregation

and regulating the activity of endogenous chaperones. It has been shown that pretreating cells with chemical chaperones 4-phenyl butyric acid (PBA) or tauroursodeoxycholic acid (TUDCA) can protect against ER stress induced cell death. In a type 2 diabetic mouse model, the *ob/ob* mice, treatment with PBA and TUDCA can alleviate ER stress, improve glucose intolerance and insulin sensitivity. (Ozcan *et al*, 2006) Here, we tested the effect of chemical chaperones in Wolfram syndrome patient fibroblasts, and observed stabilization of the mutant WFS1 protein with TUDCA and curcumin treatment. Since many Wolfram syndrome patients have point mutations in WFS1 gene encoding unstable but functional proteins, by stabilizing these proteins, we might be able to mitigate the pathological phenotypes in these patients.

In this work, we also did small-scale screening of drugs that are protective against calcium dyshomeostasis induced death. Out of the 8 positive hits we discovered, dantrolene was the most interesting one. Not only is it a FDA approved drug, but also because it is a known calcium regulator, which makes it a mechanism-based drug. Dantrolene has been proscribed as a muscle relaxant, and also studied in neurodegenerative diseases (Krause *et al*, 2004; Li *et al*, 2005; Huafeng Wei 1996), but it's application on other tissues and disorders have not been fully investigated. In this work, we propose that dantrolene could be a candidate drug for Wolfram syndrome and even other complex disorders such as diabetes, which also show dysregulation of cellular calcium.

3. Induced pluripotent stem cells transplantation

Stem cell therapy is another option for Wolfram syndrome. This year, the first clinical trial on human using induced pluripotent stem cells (iPSCs) was performed in Japan (Kamao *et al*, 2014). It was used to treat age-related macular degeneration. If the trial turns out successful, it would be good news for numerous patients. iPSCs transplantation has its advantages. First of all, it is autologous transplantation, thus inducing no immune response in patients. Secondly, the source of iPSCs is easy to obtain, it can come from any cell types, skin fibroblast being the most popular. Moreover, we can genetically modify cells before transplanting back into the patients. Lastly, unlike drugs, iPSCs could be regulated by *in vivo* signals and they respond to environmental factors, precisely producing the right amount of ingredients the body needs. Presently, insulin injection is widely used by T1D patients and some T2D patients to prevent diabetes complications. Besides being a painful and tedious process, injection of insulin needs to be carefully monitored, else, over-dosage may lead to hypoglycemia, another harmful symptom. A method that can concisely regulate the amount of insulin patients received is in urgent need (Borowiak & Melton, 2009). Recent publication showed the successful generation of functional pancreatic β cells from human pluripotent stem cells (Pagliuca *et al*, 2014). Transplantation of these cells into diabetic mice greatly ameliorated hyperglycemia and more importantly, they can secrete insulin into the serum in a glucose dependent manner. Hopefully in the near future, iPSCs differentiated β cells will be applied in clinical trials for diabetic patients. In the case of Wolfram syndrome, for patients who have milder symptoms and later onset, we can derive iPSCs into neurons and pancreatic β cells and transplant them back into the patients. For Wolfram syndrome

patients with more severe symptoms, we need to correct the mutant *WFS1* allele in the genome before transferring the cells back to the patients. Otherwise, loss of the newly transferred neurons and β cells might occur again in their lifetime. Though much progress has been made in iPSCs studies, challenges still exist. The current challenge for iPSCs treatment is the differentiation step. Methods for direct differentiation of iPSCs into specific cell types have been developed over the years, but efficiency and quality of the process still remains to be improved in the future.

ER homeostasis is a critical checkpoint for diseases

When we try to understand the cellular mechanism of a disease, the first few aspects that we tend to examine are apoptosis, mitochondrial respiration, and basic cellular functions such as proliferation defect or migration abnormality. Here we show that ER homeostasis is another critical checkpoint for diseases. The ER is the major site of protein folding, protein modification, lipid metabolism and calcium level modulation. When the ER cannot fulfill all its functions properly due to genetics defects or environmental stimuli, the balance of ER homeostasis is disrupted, and cells become unhealthy.

1. Tools developed to identify dysregulated ER homeostasis

Currently, the classical method for monitoring ER homeostasis is by determining the levels of UPR markers such as BiP, CHOP and spliced XBP1. While this remains a convincing approach, accumulation of unfolded proteins only reflects one aspect of ER homeostasis. ER homeostasis also includes the retention of ER calcium,

modulation of ER redox state, regulation of lipid metabolism, etc. Precise and feasible tools that can provide real time monitoring of the ER status are in urgent need. Previously, scientists developed an ER resident redox-sensitive GFP in yeast to dynamically measure ER redox state (Merksamer *et al*, 2008). The redox-sensitive GFP experiences conformational change under distinct pH conditions and exhibits altered emission signal intensities at excitation 400 and 490 nm. By calculating the fluorescence signal ratio from these two excitation wavelengths, we can estimate the ER redox status. Later this technology was also introduced into the mammalian system (Kanekura et al, 2013). Similar tools can also be developed to monitor ER calcium and ER lipid synthesis. One method is to establish ER calcium probes that emit distinct signals when free or bounded to calcium to visualize calcium level changes. To understand the dynamics of calcium ions, we can also use calcium radioisotope to chase calcium uptake and release in the ER. ER is also an important organelle for fatty acid synthesis and cholesterol metabolism. Scientists have already developed probes for live cell imaging of lipids, if co-expressed with an ER marker such as pDsRed ER, it would be possible to analyze ER lipid content and trafficking with a live imaging tool.

2. Modulating ER homeostasis can facilitate disease prevention and treatment

In many cases, ER homeostasis disruption is the first sign of disease symptom, and if left untreated, an array of anti-survival pathways will be activated and cells will undergo massive cell death. However, if treated properly to regain normal ER function, these cells could be rescued. What's more, we can mimic ER dysregulation status from

various diseases, or even different stages of the same disease, and screen for drugs to prevent or treat these diseases at different stages.

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