Sarco(endo)plasmic reticulum ATPase (SERCA) is a molecular partner of Wolfram syndrome 1 (WFS1) protein, which negatively regulates its expression.

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SUMMARY

Wolfram syndrome is an autosomal recessive disorder characterised by neurodegeneration and diabetes mellitus. The gene responsible for the syndrome (*WFS1*) encodes an endoplasmic reticulum (ER)-resident transmembrane protein that is involved in the regulation of the unfolded protein response, intracellular ion homeostasis, cyclic AMP production and regulation of insulin biosynthesis and secretion. In this study, single cell Ca^{2+} imaging with fura-2 and direct measurements of free cytosolic ATP concentration ($[ATP]_{CYT}$) with adenovirally expressed luciferase confirmed a reduced and delayed rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{CYT}$), and additionally, diminished $[ATP]_{CYT}$ rises in response to elevated glucose concentrations in WFS1 depleted MIN6 cells. We also observed that SERCA expression was elevated in several WFS1 depleted cell models and primary islets. We demonstrated a novel interaction between WFS1 and SERCA by co-immunoprecipitation in Cos7 cells and with endogenous proteins in human neuroblastoma cells. This interaction was reduced when cells were treated with the ER stress inducer dithiothreitol (DTT). Treatment of WFS1 depleted neuroblastoma cells with the proteosome inhibitor MG132 resulted in reduced accumulation of SERCA levels compared with wild type cells. Together these results reveal a role for WFS1 in the negative regulation of SERCA and provide further insights into the function of WFS1 in calcium homeostasis.

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INTRODUCTION

There is increasing evidence that endoplasmic reticulum (ER) perturbation plays a critical role in cell death in both neurodegenerative disorders (1,2) and diabetes mellitus (3,4). Abnormal release of calcium from the ER has been observed in several pathological conditions affecting the nervous system (5,6). ER stress, oxidative stress, palmitate, and chronic high glucose, all decrease pancreatic beta cell ER calcium levels, leading to beta cell death (7).

The ER functions as a calcium store through the expression of at least 3 types of proteins: the sarco-endoplasmic reticulum calcium ATPase (SERCA) family of proteins that actively pump calcium into the ER; luminal calcium binding proteins for storing calcium; and the gated calcium channels inositol trisphosphate receptors (IP3R) and ryanodine receptors (RyR) for the controlled release of calcium from the ER along its electrochemical gradient (8). ER calcium depletion may be associated with, among others, toxin interaction with the IP3R (GM1 gangliosidosis (9)); over-activation of the RyR (Gaucher disease (6)); SERCA inhibition (Sandhoff disease (10)) and increased SERCA expression as a compensatory mechanism through regulation by ATF6 during the ER stress response (11).

Childhood-onset diabetes mellitus and progressive optic atrophy are the diagnostic features of Wolfram syndrome, a genetic form of both diabetes and neuro-degeneration (12). In this disease, pancreatic beta cells and presumably neuronal cells are selectively destroyed due to mutations in the *WFS1* gene, which encodes WFS1 protein or Wolframin, an ER transmembrane protein (13). The mechanism is thought to be through perturbed ER homeostasis (14, 15) leading to ER stress (16,17). Loss of function mutations in *WFS1* have been shown to lead to ER calcium depletion (18), increased cytosolic calcium concentrations, and increased expression of a pro-apoptotic molecule CHOP, leading to cell death (7). Reconstitution of this ER-resident transmembrane protein into planar lipid bilayers induced a cation-selective ion channel (19).

We have previously shown that Wolframin interacts with the ion channels Na^+K^+ATPase and vacuolar-type H^+ATPase, (20,30) supporting its role in protein folding/maturation and insulin biosynthesis and secretion. In this study, while investigating the glucose signalling pathway in WFS1

depleted MIN6 cells, we observed increased SERCA protein expression. These observations prompted us to investigate the possibility of an interaction between WFS1 protein and SERCA.

RESULTS

Wolframin-depleted beta cell lines show reduced insulin secretion in response to elevated glucose or KCl-concentrations.

Wolframin has been implicated in the normal control of stimulus-secretion coupling during regulated insulin secretion *in vivo* and *in vitro* (16,17). To study the effects of Wolframin depletion on insulin secretion and Ca^{2+} signalling in β-cells, we used the MIN6 insulinoma cell line. These stable cell lines have been engineered to provide reduced *WFS1* expression using RNA interference, with 0% (wild type), 50% (KDA) or 70% (KDB) reduction in Wolframin expression achieved by expression of suitable scrambled or anti-*WFS1* shRNAs (16, 20).

Insulin release in response to glucose (30 vs 3 mmol/l) was decreased in *WFS1* depleted cells (Fig. 1A). At 30mmol/l glucose (30G) the percentage of insulin released was decreased seven-fold for KDA ($p=2.2 \times 10^{-7}$) and four-fold for KDB ($p= 4.5 \times 10^{-7}$) cells respectively in comparison to wild type. At 3mmol/l glucose (3G), and after depolarisation with 50mmol/l KCl (HK), the percentage of insulin released was also decreased in KDA and KDB cells, but only reached significance in the KDA cells.

Wolframin-depleted primary islets from *WFS1* conditional KO mice show defective insulin secretion in response to glucose.

To confirm that Wolframin is necessary for normal insulin secretion in response to high glucose we isolated pancreatic islets from beta cell selective conditional *WFS1* knock out (KO) mice, a kind gift from Professor A Permutt (16). The mice were 10-13 week old males: either *WFS1* conditional KO mice ($Wfs1^{flox/flox}$ $Cre^{cre/+}$) *or Wfs1* positive "floxed" littermates ($Wfs1^{flox/flox}$). We measured reduced Wolframin expression in protein lysates prepared from the islets by immunoblotting; beta actin (BA) was used as a loading control. The Wolframin expression was decreased by 70% in the KO islets in comparison to the controls (Fig 1B). The residual Wolframin level probably reflected the presence of WFS1 protein positive cells in these islets: all islet non-beta cells were WFS1 protein positive; *cre* recombinase was expressed from the rat insulin 2 promoter (16).

The isolation of islets and insulin secretion assay were performed as described in Materials and Methods. The percentage of insulin released in response to glucose (17 vs 3mmol/l) during static incubation for 1 h was decreased about 65% in islets from *Wfs1* KO mice in comparison to size matched control islets: percentage of insulin released by control islets (C) = 1.55±0.08%, while by KO islets= 0.54±0.15%, p=0.0006, (Fig1B). After depolarization with 20mmol/l KCl (HK) the percentage of insulin released from KO islets was also reduced in comparison to the control but did not reach statistical significance. These results confirmed the results obtained in MIN6 cells and are consistent with previous *in vivo and* the *in vitro* findings (16,17, 21).

Wolframin depletion results in a delayed and reduced glucose-induced rise in cytoplasmic free Ca^{2+}.

The above results suggested that alterations in glucose sensing may result in diminished glucose-induced insulin secretion in *WFS1*-depleted cells. To explore this possibility further, we investigated glucose- and KCl-induced cytosolic calcium ($[Ca^{2+}]_i$) rises in single, fura-2-loaded cells. The baseline $[Ca^{2+}]_i$ for each cell type was calculated as the average of the first 180 seconds of each experiment in perifusion buffer containing 3 mmol/l glucose, using the ratio of the emissions after exciting the dye at 340 and 380nm. There were no apparent differences in baseline $[Ca^{2+}]_i$ between the cell lines (340:380 ratios (F/F0): wild type cell 0.30± 0.005; KDA 0.28± 0.003; KDB 0.32± 0.004, and the F/F0 at the start of experiment was assumed to be equal 1for each cell line).

By contrast, a delayed and reduced $[Ca^{2+}]_i$ rise was observed in response to an increase in glucose concentration from 3 to 30 mmol/l in *WFS1*-depleted MIN6 cells in comparison to wild type cells; representative traces for wild type, KDA and KDB cells are shown in Fig. 2A,B,C. Quantitated by measurement of the area under the curve (AUC) following stimulation (Fig. 2D), the response in *WFS1* depleted cells was reduced 2.2- to 2.4-fold in comparison to wild type cells (AUC for wild type, KDA and KDB were: 477.4 ± 21.6; 211.4 ± 21.6; 199.1± 23.6 respectively; p values: wild type v

KDA: p=3.2 x10^{-14}; wild type v KDB: p=2.8x10^{-12} ; KDA v KDB: p=0.3; wild type n=61, KDA n=36, KDB n=21 cells).

The delay before a detectable increase in $[Ca^{2+}]_i$ following stimulation with 30 mmol/l glucose was also more than 2-fold longer in *WFS1* depleted cells than in wild type cells (Fig. 2E). Whilst the wild type cells responded after 203.7± 6.8 s, KDA and KDB cells responded only after 410.0 ± 12.9 and 416.7± 15.4 s respectively (*p* values: wild type vs KDA: p=6.2 x 10^{-18}; wild type vs KDB: p=7.3 x 10^{-16}; KDA v KDB: p=0.3). All cell lines responded similarly to depolarisation with 50 mmol/l KCl (Fig. 2A, B, C). The response to 50mmol/l KCl was quantitated by measurement of the area under the curve (AUC) and is presented on Fig. 2F (AUC for wild type, KDA and KDB were: 435.1±9.0; 447.3± 15.8, 445.1± 16.2 respectively, wild type n=61, KDA n=36, KDB n=21 cells).

Cytosolic free Ca^{2+} concentrations at baseline are not significantly different in Wolframin depleted cells compared to wild type cells.

To confirm that there was no apparent differences in baseline $[Ca^{2+}]_i$ between the cell lines we measured $[Ca^{2+}]_{CYT}$ in wild type and KDB cells with cytosolic (untargeted) aequorin. Our results (Fig.3) showed only a slightly higher level of cytosolic Ca^{2+} in KDB than in wild type cells. The results come from two independent experiments (wild type n =8; KDB n =9 separate measurements), and are consistent with our earlier findings of no significant differences in baseline $[Ca^{2+}]_i$ between the cell lines in single, fura-2-loaded cells.

Wolframin depletion results in a failure of glucose-induced rises in cytoplasmic free ATP concentrations.

To investigate the causes of the reduced and delayed cytosolic $[Ca^{2+}]$ responses to high glucose, direct measurements of free cytosolic ATP concentration were performed by expressing recombinant firefly luciferase using an adenoviral vector (22,23), and using photon-counting (24). Luciferase catalyses the formation of oxyluciferin from luciferin and ATP. The production of light, with intensity proportional to the free concentration of ATP, can be detected with a suitably sensitive photon-counting device

(25). Representative traces showing the apparent increase in free $[ATP]_{CYT}$ (as photon counts) for wild type, KDA and KDB respectively are presented in Fig. 4 A,B,C, with quantitative analysis in Fig. 4D. The increase in $[ATP]_{CYT}$ in response to high glucose was 14.1% ± 0.6 for the wild type MIN6 cells (Fig. 4A), consistent with previous results (22,23,26) but only 4% ± 1.4 for KDA ($p=7.43 \times 10^{-5}$) (Fig. 4B) and 1.3% ± 0.3 for KDB ($p= 1.8 \times 10^{-13}$) (Fig 4C). The apparent rise in $[ATP]_{CYT}$ in response to high glucose was approximately three-fold less in KDA than in wild type MIN6 cells and about ten-fold lower in KDB than in wild type MIN6 cells; (n, wt=12, n, KDA=6 and n, KDB=12 runs in three separate experiments for KDB and two separate experiments for KDA) (Fig. 4D).

To determine whether changes in the apparent $[ATP]_{CYT}$ increase in response to glucose may be due to alterations in Ca^{2+} pumping into the ER or other intracellular stores, we monitored the impact of pharmacological depletion of these stores on the observed changes (Fig. 4). Interestingly, and in contrast to previous studies (27), inhibition of SERCA pumps with cyclopiazonic acid (CPA) decreased the magnitude of the glucose-induced rise in $[ATP]_{CYT}$ in wild type cells, presumably reflecting enhanced ATP consumption for Ca^{2+} transport by other mechanisms (e.g. extrusion across the plasma membrane by PMCA (plasma membrane Ca^{2+} ATPase) or transporting into the lumen of Golgi with SPCA (secretory pathway Ca^{2+} ATPase) (Fig. 4A). By contrast, in both *WFS1* depleted cell lines, SERCA inhibition increased free $[ATP]_{CYT}$ (Fig 4B,C).

To confirm the above results we investigated ATP:ADP ratio rise to glucose using a different (static) method as described in Materials and Methods. Briefly, cells were grown in 6 well plates, starved overnight in medium with low glucose and on the day of the experiment incubated in buffers with either low glucose (3G), high glucose (30G) or high glucose and CPA (inhibitor of SERCA pump) and harvested in ice cold perchloric acid (PCA). Determination of ATP levels was performed as previously described (28) and as given under Materials and Methods and ATP:ADP ratio was calculated for all conditions. Our results show that ATP:ADP ratio rise to glucose is significantly smaller for KDB than for the control MIN6 cells: ATP:ADP ratio rise to glucose (30G vs 3G) was 1.24±0.06, 1.06±0.03 and 1.28±0.06 for the control, KDB and KDA respectively; only for KDB the difference reached the statistical significance (T test CTRL vs KDB p=0.01, CTRL vs KDA p=0.3). These results confirmed

our earlier finding that the ATP rise in response to high glucose is impaired in WFS1-depleted MIN6 cells.

Total ATP levels are reduced in WFS1 depleted cells.

We next measured total ATP content in wild type and *WFS1*-depleted MIN6 cells as described in Materials and Methods. The results were normalised for the cell number and presented as Relative Luminescence Units (RLU, proportional to the amount of ATP) per 10^4 cells, (Table 1). The total levels of ATP (RLU) were significantly reduced in both WFS1 depleted cell lines to 79% (of the wild type) in KDA and 53% in KDB.

SERCA expression is increased in WFS1 depleted cells.

The decreased levels of cytosolic ATP could have been the result of an ATP generation defect or ATP overconsumption in WFS1 depleted cells. As no defect in oxidative phosphorylation was identified previously in Wolfram patient biopsies (29), there was a possibility that ATP may be over-consumed in WFS1 depleted cells. One of the major ATP consumers in pancreatic cells is the SERCA calcium pump. We hypothesised that SERCA over-activity could result in ATP depletion in WFS1 depleted cells and investigated the levels of SERCA expression.

We examined SERCA expression levels in several WFS1 depleted cell models: MIN6 pancreatic cells, primary pancreatic islets isolated from conditional *Wfs1* KO mice and in WFS1 depleted SK-N-AS neuroblastoma cells (Fig 5). All the WFS1 depleted models displayed Unfolded Protein Response (UPR) activity, measured as an increase in the levels of ER stress markers (16, 30).

To examine SERCA expression in MIN6 cells we prepared extracts from microsomal fractions from control and WFS1 depleted KDA and KDB cell lines (31) and examined the levels of SERCA expression by immunoblotting using PanSERCA antibody Y1F4 (32). Protein disulfide isomerase (PDI) was used for normalization. In both WFS1 depleted cells KDA and KDB the SERCA expression was increased over two fold in comparison to WFS1 positive control (KDA=287.1±82.2, p=0.045, and KDB=282.1±83.0, p=0.052 n=6 for both, C=100%, Fig 5A).

To examine the SERCA expression levels in isolated islets we prepared total protein extracts from 10-13 weeks old *Wfs1* conditional KO males as described in Materials and Methods. There was a greater than 50% increase of SERCA levels in *Wfs1* conditional KO islets in comparison to *Wfs1* positive controls: (C=100%, KO=157.8±17.8, p=0.005, n=4 Fig 5B). Wolfram syndrome manifests also as a neurodegenerative disease. Therefore we used three WFS1-depleted neuroblastoma cell lines (KD1-KD3) depleted by 60-80% in comparison to the control (C) as described by Gharanei et al., (30) and examined the SERCA expression using either Pan-SERCA antibody Y1F4 (Fig 5C) or isoform SERCA2 specific antibody (Fig 5D). The expression levels of SERCA were increased in all WFS1-depleted cell lines. Fig 5C shows the increased expression levels detected with Pan SERCA antibody (KD1=261.8%± 30.8, p=0.02, n=3; KD2=271.2±42.9%, p=004, n=8; KD3=233.7±40.3%, p=0.009, n=8, C=100%). We confirmed that this increase is mainly due to an increase in SERCA2 expression. Fig 5D shows increased levels of isoform SERCA2 (KD1=189.8±28.6%, p=0.04, n=4; KD2=318.9±71.2%, p=0.02, n=6; KD3=301.7±59.3%, p=0.02, n=6, C=100%).

In summary, all WFS-depleted models showed increased levels of SERCA expression, (the main contributor being isoform SERCA2). This is consistent with our hypothesis that in WFS1-depleted cells SERCA may be overactive.

WFS1 interacts with SERCA2.

To explain how the absence of WFS1 protein may affect the levels of SERCA expression we hypothesized that the two proteins may be molecular partners. We first examined the potential WFS1-SERCA2 interaction in an over-expression system. We co-transfected FLAG-SERCA2 plasmid (constructed as described in Materials and Methods) together with Myc-WFS1 (20) or with the empty vectors to Cos7 cells. The following protein extracts were used: Myc-WFS1/FLAG-SERCA2, Myc-WFS1/empty FLAG, empty Myc/FLAG-SERCA2 and empty Myc /empty FLAG for immunoprecipitation with anti FLAG antibody (Fig. 6A). Input panels at the left hand side show the expression of relevant proteins in extracts before co-immunoprecipitation. Immunoprecipitation panels at the right hand side show co-immunoprecipitation with mouse monoclonal anti FLAG antibody probed with either anti c-myc (top panel) or anti WFS1 antibody (middle panel). The results on Fig 6A

show that with anti FLAG antibody we were able to precipitate Myc-WFS1 (lane1). No WFS1 was detected in lanes 2-4, where either Myc-WFS1/empty FLAG (lane2), empty Myc /FLAG-SERCA2 (lane3) or empty Myc /empty FLAG (lane 4) plasmids were present. We probed the membrane with polyclonal rabbit anti FLAG antibody (bottom panel) to show the input.

In a reciprocal experiment (Fig 6B, right hand side panel) polyclonal rabbit anti c-myc antibody was used for co-immunoprecipitation and monoclonal mouse anti FLAG antibody for detection (top panel). Only in lane1, where both Myc-WFS1 and FLAG-SERCA2 proteins were expressed, we observed co-immunoprecipitation of FLAG-SERCA2. There was no SERCA2 detected in lanes 2-4, where either Myc-WFS1/empty FLAG (lane2), empty Myc/ FLAG-SERCA2 (lane3) or empty Myc /empty FLAG (lane4) were present. The bottom panel probed with monoclonal mouse c-myc antibody shows the input. These results indicate that WFS1 and SERCA2 can interact in an over-expression system.

Next we examined if endogenous WFS1 and SERCA2 can be co-immunoprecipitated from SK-N-AS neuroblastoma cells (Fig 6C). WFS1 antibody (rabbit polyclonal) were used for precipitation (lane5) and a ~110 kDa band was detected with SERCA2 antibody. No SERCA2 was detected in either lane 4 or 6 where negative controls: either FLAG- rabbit polyclonal or FLAG -mouse monoclonal antibody were used for co-immunoprecipitation. Our results indicate that the two proteins, WFS1 and SERCA2 may interact with each other in SK-N-AS neuroblastoma cells.

To understand the role of this interaction we examined if WFS1 and SERCA2 can interact with each other under conditions of ER stress. We prepared protein extracts from neuroblastoma SK-N-AS cells either untreated or treated with ER stress inducer: 1mmol/l DTT for 3 hours. First, we examined the expression of both proteins in these extracts (Fig 6D, left panel): the expression levels of SERCA2 were slightly elevated in DTT treated extract (D) in comparison to untreated (U) (D: 134.8±8.1%, U:100%, $p=0.02$, $n=4$), WFS1 levels were unchanged by the treatment: (D: 97.8±6.3%, U=100%, $p=0.75$, $n=4$). In summary, there was no decrease in the expression of either of these two proteins upon DTT treatment (Fig 6D, left panel).Then, we performed co-immunoprecipitation of SERCA2 with rabbit WFS1 antibody (Fig 6D, right panel, $n=3$) using either DTT treated extract (lane 3), or untreated extract (lane 4). We detected the presence of SERCA2 band in lane 4 (top panel), where

untreated extract was used. Interestingly, the SERCA2 band in the DTT treated sample was much reduced (Fig 6D, lane3, top panel). The membrane was re-probed with sheep WFS1antibody and the amount of precipitated WFS1 quantified showing equal amounts in both DTT treated and untreated samples (DTT:1.0, Untreated:1.0, Fig.6D, lower panel). Next, the amount of precipitated SERCA2 was quantified, and normalized for the WFS1 levels: (untreated sample: SERCA2 =1.0; DTT treated sample: SERCA=0.38). In summary, DTT treatment did not affect either the protein expression or the amounts of precipitated WFS1 (Fig 6D). This suggests that the 62% reduced levels of the amount of precipitated SERCA2 in DTT-treated sample do not result from reduced levels of precipitated WFS1 under these conditions. Our results thus suggest that WFS1-SERCA2 interaction may be impaired under ER stress conditions.

Proteasome inhibition results in an increase of SERCA2 levels in a WFS1-dependent manner.

We demonstrated above that WFS1 interacts with SERCA and SERCA expression is increased in WFS1 depleted cells. We hypothesized that WFS1 may be a negative regulator of SERCA and targets SERCA to proteasome mediated degradation. To test our hypothesis we compared SERCA levels in control and the WFS1 depleted cell line KD2 after treatment with the proteasome inhibitor MG132. Control and WFS1 depleted KD2 cells were treated with either 5 or 10µmol/l MG132 proteasome inhibitor as described by Gharanei et al., (30). Equal amounts of protein (11µg) was loaded per lane and samples were resolved on SDS PAGE gels. SERCA expression in DMSO treated sample was assumed to be equal 100%. Quantification revealed that while in control cells SERCA expression at 5µmol/l increased to 226.9± 26.5% and at 10µmol/l increase to 266.3±51.9% (2.2 and 2.6 fold respectively, n=6) the increase of SERCA expression was much reduced in KD2 cells to 125.0±8.9% at 5µmol/l and 106.7±24.3% at 10µmol/l (1.2 and 1.1 fold respectively, n=4). The difference in accumulation of SERCA between control and KD2 cells was statistically significant: C v KD2 at 5µmol/l: p=0.01; C v KD2 at 10µmol/l: p=0.03, n=6, (Fig 7). This result, together with the previously demonstrated interaction between the two proteins and significantly increased SERCA levels in WFS1 depleted cell model is consistent with the possibility that WFS1 targets SERCA to the proteasome for

degradation. However, the ubiquitnation assay, in which we compared SERCA ubiquitnation levels between KD2 and control cells did not show impaired SERCA ubiquitination in KD2.

DISCUSSION

In Wolfram syndrome, diabetes mellitus results from multiple defects in the glucose signalling pathway; and both the diabetes mellitus and neurodegeneration are thought to be associated with ER calcium depletion. The present study provides additional insights into its role in both mouse pancreatic beta cell and human neuroblastoma cell models of the disease. We observed the following: (1) our models confirmed that WFS1 protein depletion in MIN6 beta cells and primary islets resulted in reduced glucose stimulated insulin secretion, and reduced and delayed cytosolic Ca^{2+} rise to glucose; (2) we observed a reduced cytosolic ATP rise in response to high glucose, and reduced total cellular ATP content; (3) SERCA expression levels were increased in WFS1 protein depleted MIN6 cells, WFS1 KO primary islets, and WFS1 protein depleted human neuroblastoma cells; (4) WFS1 protein interacted with SERCA in both overexpressed and endogenous models; (5) Proteasomal inhibition studies suggested a role for WFS1 in SERCA protein turnover. (6) When ER stress was induced with DTT, there was a reduced WFS1/SERCA complex formation or partial dissociation, allowing SERCA expression to increase. These results are consistent with a model in which WFS1 protein negatively regulates the ER stress response and modulates ER calcium filling by regulating SERCA expression to partially compensate for ER calcium depletion in conditions of ER stress.

We showed that in two MIN6 β-cell models of Wolframin depletion there was reduced glucose stimulated insulin secretion (Fig 1A). We also demonstrated the defect in glucose stimulated insulin secretion in primary islets isolated from conditional beta cell specific knock out mice (Fig.1B). In our MIN6 model of Wolframin depletion, this was associated with a delayed and reduced amplitude of the $[Ca^{2+}]_i$ rise (Fig 2), and reduced $[ATP]_{CYT}$ rise in response to elevated glucose (Fig 4). These findings thus confirm and extend previous observations of multiple defects in the control of glucose signalling and insulin secretion in Wolframin depleted β cells (16,17,21).

Our findings of reduced $[ATP]_{CYT}$ rise in response to elevated glucose, and reduced total cellular ATP, suggest reduced ATP synthesis or increased ATP consumption after WFS1 silencing (22,27). This reduced $[ATP]_{CYT}$ rise may explain the observed reduced and delayed glucose-induced $[Ca^{2+}]_i$ rise in these mutants (due to failed closure of ATP-sensitive K^+ channels). ATP generation defects have not been previously reported in Wolfram syndrome; our own previous work demonstrated normal oxidative phosphorylation in muscle biopsies from Wolfram patients (29). Increased ATP consumption could arise from cellular efforts to restore ER homeostasis. SERCA pump activity has been shown to be a major ATP-consuming process in MIN6 cells (27) and has also been proposed as a negative regulator of $[ATP]_{CYT}$, contributing to the Ca^{2+}-dependent oscillations in $[ATP]_{CYT}$ observed in beta cells (33,34).

These observations led us to hypothesise that ATP utilisation by SERCA may increase in the absence of WFS1 protein; and further, that increased activity/expression of SERCA would be observed in WFS1 protein depletion. To test our hypothesis we studied the levels of SERCA expression in several models of WFS1 depleted cells: pancreatic MIN6, SK-N-AS neuroblastoma and in primary islets isolated from *WFS1* conditional knock out mice. All our models showed elevated ER stress markers (16,30). We found that SERCA levels were significantly elevated in all our models and that it was mainly due to increased levels of isoform SERCA2 (Fig 5). This is a novel finding and to the best of our knowledge, there are no published reports of SERCA expression in WFS1 protein depleted cell models.

We demonstrated that WFS1 and SERCA interact in both an over-expression system (Fig 6AB) and endogenously (Fig 6C); and that this interaction is reduced in the presence of DTT-induced ER stress (Fig 6D). Inhibition of the proteasome with MG132 resulted in accumulation of SERCA in a WFS1 dependent manner: the accumulation of SERCA expression was reduced in WFS1 depleted neuroblastoma cell line KD2 in comparison to WFS1 positive control (Fig 7). These results suggested that WFS1 my be a negative regulator of SERCA, and were consistent with the possibility that the WFS1-SERCA interaction regulates SERCA levels by targeting SERCA to degradation by the proteasome. Our ubiquitination study did not confirm impaired SERCA ubiquitination in WFS1

depleted cells (data not shown). We tested for "canonical " signals for proteasomal recognition with antibody against ubiquitin lysine-48 based chains. However, several "non canonical" ubiquitin based signals for proteasomal targeting have also been described (e.g. polyubiquitin chains assembled through residues other than lysine 48), tagging substrates with "ubiquitin like" proteins, or proteasomal degradation without prior ubiquitination (35). Therefore the mechanism of WFS1 regulation of SERCA requires further confirmation.

It seems that the regulation of SERCA protein levels by WFS1 may be ER stress dependent: under conditions of ER stress SERCA is released from the interaction with WFS1 (or alternatively SERCA-WFS1 complexes are formed at a lower rate), it may be degraded by the proteasome to a lesser extent, and SERCA expression increases. Under normal conditions of ER homeostasis the physiological levels of SERCA may be maintained in homeostasis by interaction with WFS1. A similar function for WFS1 was described by Fonseca et al., (15) who demonstrated that WFS1 negatively regulates a key transcription factor involved in ER stress signalling Activating Transcription Factor 6α (ATF6α) through the ubiquitin-proteasome pathway.

Some forms of ER stress result in ER calcium depletion, disturbed ER functions and increase in cytosolic calcium levels which may trigger cell death via activation of the calpain-2 apoptotic pathway (7). We provide evidence that under conditions of WFS1 protein depletion, there may be a secondary increase in SERCA expression to pump calcium ions back into the ER calcium store to try to restore ER homeostasis. Several reports show that induction of ER stress may lead to increased SERCA expression on both mRNA and protein levels (36,37,38,11,39). In addition Wu et al., (40) reported that cytosolic calcium elevation itself increased SERCA2 expression by a mechanism distinct from ER stress. The authors speculated that enhanced calcium uptake into the ER might shorten the period of a relative ER calcium depletion subsequent to a stimulus–induced ER calcium release, in this way responding to an increased demand for calcium by ER chaperones system under the conditions where unfolded proteins can be accumulated. In contrast, other authors have reported that under pathological conditions associated with cell death, including ER stress, SERCA expression is decreased (7,41,42). The discrepancy may relate to the degree of ER stress: in resolvable ER stress, SERCA expression and

function may increase to restore ER calcium levels; in irresolvable ER stress, ER calcium levels cannot be restored SERCA expression falls and apoptosis ensues.

The absence of WFS1 is associated with ER stress (14,15,16,17), calcium leak from the ER and an elevation of cytosolic calcium levels (7). ER stress, ER calcium leak and elevations of cytosolic calcium have all been associated with increases in SERCA expression (11,36,37,38,40). ATF6 is also negatively regulated by WFS1, and a downstream effect of increased ATF6 activity is elevation of SERCA expression (11). It may be that under these conditions for the cells to survive SERCA expression must be increased to counteract the ER calcium leak to restore ER calcium homeostasis and to prevent activation of cell death pathway induced by elevated cytosolic calcium levels. The decrease of SERCA levels under ER stress and under some pathological conditions observed by Hara et al., (7) was in the presence of WFS1, where induction of this compensatory mechanism was not necessary; WFS1 exerted its pro-survival function and prevented cell death. Our results, summarised on Fig 8, are consistent with the possibility that WFS1 negatively regulates SERCA turnover, possibly via proteasome mediated degradation and this process is dependent on the ER stress. Release of SERCA from interaction with WFS1 in WFS1 depleted cells or under ER stress may result in increased SERCA levels and activity and may allow compensatory pumping of calcium ions from cytosol to ER lumen to restore ER calcium homeostasis and prevent cell death.

To further understand WFS1 role in calcium homeostasis it will be interesting to examine if/how WFS1 depletion affects the expression of inositol trisphosphate (IP3) receptors and ryanodine receptors (RYR), the calcium channels that release calcium from the ER; and how WFS1 depletion affects other intracellular Ca^{2+} stores.

In summary, our results support previously reported WFS1 involvement in regulation of glucose sensing and insulin secretion in pancreatic cells and demonstrate for the first time a defective ATP response to glucose and ATP depletion in the absence of WFS1. We also demonstrate that WFS1 is an interactive partner with and a negative regulator of SERCA.

Materials and Methods.

Cell culture and adenoviral infection

Mouse β-cell-derived MIN6 cells expressing interfering RNAs to provide reduction in *WFS1* expression (16) were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 25mmol/l glucose, 2mmol/l pyruvate, supplemented with 15% (vol/vol) FBS, 4mmol/l glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 143 µmol/l β-mecaptoethanol and 0.2 mg/ml geneticin. Luciferase expression was achieved by infection of cells with adenoviral vector encoding cytoplasmic-targeted luciferase (AdCMVcLuc) (22), aequorin expression by using untargeted cytosolic aequorin (Cyt.Aq) (43). Human neuroblastoma SK-N-AS cells with stable WFS1 depletion were grown as described by Gharanei et al., (30).

Insulin secretion from MIN6 cells.

Cells were seeded in six-well plates and grown in full growth medium for at least 16 hours, before they were maintained overnight in medium with 3mmol/l glucose. Insulin secretion was performed as previously described (http://www.jbc.org/content/280/27/25565.long) and insulin content measured by radioimmunoassay (Millipore).

Isolation of pancreatic islets, insulin secretion assay and preparation of protein extracts.

The mice used in this experiment were beta cells selective conditional *Wfs1* knock-out mice (16), obtained by collaboration from Professor A. Permutt. They were additionally back crossed with C57 wild type females for two generations and the heterozygotes were used as parents in matings to obtain experimental and control littermates. Islets were isolated by collagenase digestion from pancreata of 10-13 weeks old male mice: either *Wfs1* conditional knock out mice ($Wfs1^{fl/fl}$ $Cre^{cre/+}$) or *Wfs1* positive "floxed" littermates ($Wfs1^{fl/fl}$). Islets of Langerhans were isolated, cultured and insulin secretion assay performed as previously described (26,44).

For preparation of protein lysates for immunoblotting the islet were incubated in RPMI medium for 10-12 days, hand picked every other day and lysed in RIPA buffer (400-500 islets were lysed in 60-70

μl of buffer). For homogenization the lysate was multiple times frozen (at -80C) and defrosted at RT, each time being passed several times through pipette tip followed by passing through a 21.5G size needle (26,44).

Cytosolic free Ca^{2+} concentration measurements with fura-2

Cells were seeded on 24 mm diameter coverslips and starved in medium containing 3mmol/l glucose overnight. The next day, cells were loaded with the fluorescent dye fura-2 (5μmol/l), and incubated in Krebs buffer (see above) with 3mmol/l glucose. Next, the cells were perifused with buffer containing 3mM glucose followed by 30mmol/l glucose, then 50mmol/l KCl as a control. Changes in ($[Ca^{2+}]_i$) in single cells were measured as the changes in fluorescence intensity of fura-2 using an Olympus IX-80 inverted optics epifluorescence microscope (x40 oil immersion objective). Single cell $[Ca^{2+}]_i$ measurements were performed exciting the dye at 340 and 380nm and emission was recorded at 510nm. Images were recorded with an IMAGO charge-coupled device camera (Till Photonics GmbH, Grafelfing, Germany) controlled by Tillvision software (Till Photonics).

Measurements of cytosolic free Ca^{2+} with recombinant targeted aequorin.

Cells were seeded onto 13 mm diameter coverslips and 24 h later, at 70-80% confluency, transfected with adenoviruses expressing the untargeted (cytosolic) bioluminescent protein aequorin (45,46). Forty eight hours later, cells were depleted of Ca^{2+} by incubation with 10μmol/l ionomycin, 10μmol/l cyclopiazonic acid (CPA) and 10μmol/l monensin in modified Krebs-Ringer bicarbonate buffer containing glucose 3mmol/l, Hepes 10mmol/l, KCl 3.5mmol/l, $MgSO_4$ 0.5mmol/l, NaCl 140mmol/l, $NaHCO_3$ 2mmol/l, and NaH_2PO_4 0.5mmol/l (to achieve pH 7.4), and supplemented with EGTA 1mmol/l for 5 min at 4C (46). Cytosolic free calcium levels were measured using a cytoplasmic (non targeted) aequorin construct and native coelenterazine (47). Aequorin was reconstituted with 5μmol/l coelenterazine in Krebs buffer with 0.1mmol/l EGTA for 2h at 4°C (48). The cells were perifused with Ca^{2+}-free buffer, followed by addition of buffer containing 1.5 mmol/l $CaCl_2$ at 120s to permit Ca^{2+} entry. The photon counts were detected with a photomultiplier device (Thorn EMI) (49) adjacent

to the perifusion chamber and converted to Ca^{2+} concentration [μmol/l] using algorithms described previously (50,51).

Cytosol free ATP concentration measurements

Cells were seeded onto 13 mm diameter coverslips and 24h later, at 70-80% confluency infected with adenovirus expressing untargeted (cytosolic) firefly luciferase (22,23). After 48h culture, the cells were "starved" overnight in medium containing 3mmol/l glucose. The following day, the cells were incubated for a further 15 min. in modified Krebs buffer containing 3mmol/l glucose, then perifused in the same buffer containing 3mmol/l glucose, followed by buffer with 30mmol/l glucose and then followed by addition of CPA (10μmol/l), an inhibitor of SERCA. The cytosolic free ATP concentration ($[ATP]_{CYT}$) was estimated by counting the emitted photons with a photomultiplier as described above.

Measurements of ATP: ADP ratio in response to rise in glucose concentration.

Cells were seeded in 6 well plates in standard media. The next day the cells were starved overnight in the medium with 3mmol/l glucose. On the day of experiment the cells were incubated for 15 min in Krebs buffer with 3mmol/l glucose; and after that they were incubated for 15 min in Krebs buffer with either 3mmol/l glucose (plate1), 30mmol/l glucose (plate2) or 30mmol/l glucose and CPA (plate3). The plates 1 and 2 were harvested after 15 min in 200μl per well of 20% (v/v) ice cold perchloric acid (PCA) and rapidly frozen in -80°C. 10μmol/l CPA was added to plate 3 and incubated additional 5 min before being harvested in PCA as above. In parallel, another plate was seeded in identical way for measurements of protein concentration to normalise the results and harvested in protein lysis buffer (RIPA). Standard curve for ATP and ADP (a negative control) was prepared (gradient of concentrations versus luminescence). The samples (harvested in PCA) were neutralised to pH7.4 with a known volume of neutralisation mixture (0.5mol/l triethanolamine, 2mol/l KOH and 100mmol/l EDTA). The samples were mixed with an aliquot of a reaction buffer containing sodium arsenate (65mmol/l, pH7.4), MgSO4 (20mmol/l), KCl (5mmol/l) and phosphoenolpyruvate (PEP). For each sample two assays were performed: set1=ATP measurment with "mock conversion" of ADP (in the reaction buffer as stated above, without pyruvate kinase) and set2 =ATP + ADP measurement

where ADP was converted to ATP by addition of pyruvate kinase. After two hours incubation at RT, 10µg of luciferase and 1mmol/l luciferin were added to each sample and luminescence was measured on luminometer for both: set1 (ATP) and set2 (ATP+ADP). The amount of ADP was calculated as a difference ADP = (ATP+ADP)-ATP (28).

Total cellular ATP content

Cells were grown to 90% confluency in standard medium (see above) with 25mmol/l glucose, harvested, diluted 10x in growing medium, counted and seeded in opaque-walled 96 wells plates (Appleton Woods). The following day total ATP assay was performed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Briefly, the plates were equilibrated to room temperature for 30 min, and a volume of reconstituted CellTiter-Glo Reagent (100µl to 100µl of medium in each well) was added. The content was mixed for 2 min. in an orbital shaker to induce lysis; after 10 min incubation in room temperature the luminescence was read on a multilabel counter Wallac 1420 Victor3 (Perkin Elmer).

Preparation of microsomal fractions.

8 x T75cm flasks of each: wt, KDA and KDB MIN6 (80% confluent) were harvested by trypsinisation, rinsed with PBS and resuspended in 2mls of PBS on ice. Cells were spun down at 2000 rpm, 5 min, 4ºC and suspended in 10mls of MEMBRANE buffer (5mM HEPES, 0.32M sucrose pH7.2 with protease inhibitors) before they were homogenised using electric homogeniser Polytron, ultra Turro T8 at 30 000rpm for 10 sec. Then, cells were transferred to glass electric homogeniser and homogenised with 10 strokes. Cells were spun down at 10000g, 10min, 4ºC to remove organelles (nuclei and mitochondria). Cloudy supernatant (15mls) was harvested to a separate tube, divided between two ultracentrifuge tubes (3/4 full) and spun down at 100 000x g for 1 hour at 4˚C. Pellet (microsomes) was harvested by pouring off supernatant and resuspending in 500µl each in MEMBR ANE buffer, no proteases cocktail. Aliquots of 100µl were frozen in liquid nitrogen and transferred to -70 deep freezer immediately (31).

Proteasome inhibition assay.

Cells were plated at 5×10^5 cells/well in a 6-well plate. After 24h, cells were treated with either 5 or 10µmol/l MG132 proteasome inhibitor for 4h, harvested in RIPA buffer and prepared for Western Blotting as described by Gharanei et al., (30).

Immunoblotting.

Antibody were diluted in 5% milk in PBS-Tween and used at the following concentrations: PanSERCA Y1F4 (mouse monoclonal (32)) 1:5000; SERCA2 (goat polyclonal Santa Cruz) 1:1000; Beta actin (mouse monoclonal Sigma) 1:14000; PDI (mouse monoclonal Abcam) 1:5000. For protein detection in extracts from isolated islets the following concentrations of primary antibody were used: WFS1 (rabbit polyclonal, obtained by collaboration (20)) 1:500; PanSERCA Y1F4 1:500, beta actin 1:3500. Secondary antibody (anti rabbit, goat and mouse, Dako) were used at 1:20000.

Co-immunoprecipitation.

Construction of FLAG-SERCA2 plasmid: full length human SERCA2 (transcript variant b, GeneBank NM_170665.3) was amplified from a human cDNA library by PCR using primers 5'-CTTGCGGCCGCGATGGAGAACGCGCAC-3' (forward) and 5'-GCATGGTACCTCAAGACCAGAAGATATCG-3' (reverse) and was cloned between the Not I and Kpn I site of pFLAG-CMV4 expression vector (Sigma). The sequence was confirmed by DNA sequencing. pCMV-Myc-WFS1 plasmid was described before (20).

Transfection: Cos7 cells were seeded at 2.0 x 10^6 in 10cm plates and after 24h co-transfected with the following combinations of plasmids (2µg of each): pCMV-Myc-WFS1/FLAG-SERCA2, pCMV-Myc-WFS1/empty FLAG, empty Myc /FLAG-SERCA2 and empty Myc/empty FLAG using Turbofect transfection reagent (Thermo Scientific) according to manufacturer instructions. After 48h the cells were harvested in 600 µl of RIPA buffer (20), sonicated 3x10s and centrifuged at 23000 x g at 4°C.

Immunoprecipitation in over-expression system: 750µg of protein extract and either anti-FLAG mouse monoclonal antibody (Sigma) or anti c-myc rabbit, polyclonal antibody (Sigma) were used for co-immunoprecipitation in the over-expression system. The extracts were incubated with antibody overnight at 4°C with end to end rotation. Protein G Sepharose beads were added after overnight incubation for a further 4 hours of end to end rotation at 4°C. Beads were separated from lysate using Spin-X columns (Costar) by centrifugation at 4000 x g for 15 min at 4°C and washed 4 times with RIPA buffer (for washes:1, 2 and 4 RIPA buffer with 150mmol/l NaCl was used, for wash 3 RIPA buffer with NaCl increased to 500mmol/l was used). Each wash was performed by 15min incubation at 4˚C with end to end rotation, followed by spin at 4000 x g. Bound proteins were eluted in 50µl sample buffer by heating to 70°C for 8 min followed by 3min spin at a maximum speed in microfuge and stored in -80°C. The following antibody were used for immunoblotting: c-myc (rabbit polyclonal, Sigma) 1:10000; WFS1 (rabbit polyclonal, Proteintech) 1: 500; FLAG (rabbit polyclonal Sigma) 1:1000; FLAG (mouse monoclonal, Sigma) 1:1000; c-myc (mouse monoclonal Sigma) 1:1000. Secondary anti mouse and anti rabbit antibody (Dako) were used 1:20000.

DTT treatment. 80% confluent SK-N-AS cells growing in 10cm dishes were treated with 1mmol/l DTT (Sigma) for 3 hours or remained untreated before being harvested in 600µl TBD (composition below). Equal amounts of untreated and DTT treated protein lysates were used for co-immunoprecipitation as described below.

Immunoprecipitation of endogenous proteins: To detect endogenous interaction neuroblstoma SK-N-AS cells in 10cm dishes were harvested in 600µl Tris buffer (TBD) per dish (20mmol/l Tris pH7.5, with 100mmol/l NaCl, 1%TritonX-100, 1mmol/l DTT, 1mmol/l PMSF and protease inhibitors (Complete Mini, Roche)), scraped on ice, placed in eppendorfs and incubated at 4˚C with end to end rotation for 1 hour. The samples were sonicated 3x10s on ice and the lysates cleared by centrifugation at 23000 x g at 4˚C. 900µg-1mg of protein extract (400µl) was used per reaction. WFS1 rabbit polyclonal antibody (Abcam) were used for co-immunoprecipitation and FLAG rabbit , polyclonal antibody (Proteintech) or FLAG mouse monoclonal antibody (Sigma) were used as negative controls. After overnight incubation with the antibody, the lysates were transferred to Spin-X

columns (Costar) with 20µl of pre-cleared Protein G Sepharose beads, for 3h incubation at 4˚C with end to end rotation. The samples were centrifuged at 4000 x g at 4˚C and the beads washed 2x in 400µl of buffer TBD-150 (with 150mmol/l NaCl), 1x in TBD-500 (with 500mmol/l NaCl), and again 1x in TBD-150. Each time the beads were incubated 15-20 min at 4°C with end to end rotation. Finally beads were separated from lysates by centrifugation at 4000xg and 50µl of SDS loading buffer was added per sample. The proteins were released from beads by heating to 70˚C for 8 min, spun down at 13000rpm in microfuge for 3 min and supernatant harvested. The samples were run on SDS-PAGE gels, transferred to membranes at (90V for 90 min) and boiled for 4 minutes in water before being blocked in 5% milk in PBS-Tween for 1 hour. The membranes were incubated with SERCA2 goat polyclonal antibody (Santa Cruz) 1:1000 followed by anti goat secondary antibody (Dako) 1:20000, or WFS1 sheep polyclonal antibody (R&D systems) 1: 10000 followed by secondary anti sheep (R&D systems) 1:30000 overnight at 4˚C with rotation.

Statistical analysis

Data are given as the means ± S.E. for the number of experiments given. Comparisons between means were performed using Student's t-test with Bonferonni correction for multiple testing, as appropriate.

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Conflicts of interest

The corresponding author confirms on behalf of all authors that there are no conflicts of interest to declare.

TABLE AND LEGEND

Table 1. Total ATP content (RLU) of wild type MIN6 and WFS1 depleted MIN6 cells.

Total ATP content was assayed using CellTiter-Glo Luminescent Cell Viability Assay (Promega) as described under Material and Methods. The results are presented as relative luciferase units (RLU) per 10^4 cells and as a per cent of ATP content in wild type. The results come from 3 independent experiments with n wt =72, n KDB=66, n KDA=72 (t-test: wt vs KDB p=4.8 x 10^{-21} ; wt vs KDA p=1.0 x 10^{-8}).

Table 1

Cell line	RLU/10^4 cells ± SE	% (total ATP of wt)
pSuper (WT)	378691.2± 9283	100%
KDA	297797.4± 10111	78.6%
KDB	198801.5± 12976	52.5%

FIGURE LEGENDS

Fig. 1. Glucose and depolarisation-induced insulin secretion from MIN6 cells and isolated pancreatic islets.

A. Insulin secretion from wild type and WFS1-depleted MIN6 cells. Quantitative analysis of insulin secreted in response to 30 mmol/l glucose and 50 mmol/l KCl. Total and released insulin were measured by radioimmunoassay as given under Materials and Methods. Wolframin depletion resulted in a reduction of the percentage of insulin released in response to 30 mmol/l glucose (***: wt vs KDA p=2.2 x10^{-7}, and wt vs KDB p= 4.5 x10^{-7}). The results were from three experiments performed in triplicate. 3G: 3 mmol/l glucose, 30G: 30 mmol/l glucose, HK (high potassium): 50 mmol/l KCl.

B. Glucose induced insulin secretion from primary islets isolated from conditional, beta cell selective *Wfs1* KO mice**.** Immunoblot – 70% reduced WFS1 expression in islets isolated from *Wfs1* KO mice in comparison to the control islets; 10µg of protein extract was loaded per lane. BA: beta actin. Bar chart: Quantitative analysis of insulin secreted in response to 17 mmol/l glucose and 20 mmol/l KCl measured by radioimmunoassay. The percentage of insulin released in response to 17 mmol/l glucose is significantly reduced in islets from KO mice in comparison to size matched control islets (C): (***: C vs KO p=0.0006). The percentage of insulin released in response to high potassium (20 mmol/l) is also reduced in islets from KO mice, but it did not reach statistical significance (C vs KO p=0.22). No difference was measured in response to 3mmol/l glucose (C vs KO p=0.21). The results come from at least three experiments in duplicates (for control islets (C) N=3, for KO islets N=6). 3G: 3 mmol/l glucose, 17G: 17 mmol/l glucose, HK (high potassium): 20 mmol/l KCl.

Fig. 2. Changes in cytoplasmic free Ca^{2+} concentration in response to 30 *vs* 3.0 mmol/l glucose, or 50 mmol/l KCl in wild type and Wolframin-depleted MIN6 cells. Representative traces of cytosolic free Ca^{2+}concentration changes in response to 30 (*vs* 3) mmol/l glucose for **A**: *wt* MIN6; **B**: KDA; **C**: KDB cells. The y axes (F/F0) represent the apparent free cytosolic [Ca^{2+}] as given by the normalised fluorescence intensity ratio of fura-2 upon excitation at 340 and 380nm. Note the delayed and reduced rise in $[Ca^{2+}]_{CYT}$ in response to 30 mmol/l glucose in WFS1-depleted cells. 3G: 3 mmol/l

glucose, 30G: 30 mmol/l glucose, HK (high potassium): 50mmol/l KCl. **D.** Quantitative analysis of the increase in free cytosolic Ca^{2+} concentration (fura-2) to 30 mmol/l glucose measured as AUC (area under curve). The response in WFS1 depleted cells was more than 2-fold smaller than *wt* (***: wild type vs KDA: p=3.2 x 10^{-14}, wild type vs KDB: p=2.8 x 10^{-12} , and KDA vs KDB: p=0.3). **E.** Delayed response of cytosolic calcium rise to 30 mmol/l glucose in Wolframin-depleted cells. The delay before a detectable response was more than 2.0 fold longer in WFS1 depleted cells than in wild type cells. (***: wild type vs KDA: p=6.2x10^{-18}; wild type vs KDB: p=7.3 x 10^{-16} and KDA v KDB: p=0.3; n, wild type=61; n, KDA=36; n, KDB=21 cells). **F.** Quantitative analysis of the increase in free cytosolic calcium concentration (fura-2) to 50mmol/l KCl measured as AUC; there was no differences between WFS1 depleted cells and WT MIN6.

Fig. 3. Changes in cytosolic free calcium concentration in wild type MIN6 and Wolframin depleted KDB cells. Cells were infected with adenovirus expressing untargeted (cytosolic) aequorin. 48 h later, cells were depleted of calcium (see Materials and Methods), then perifused in nominally Ca^{2+}-free buffer, followed by buffer with 1.5mmol/l $CaCl_2$ as indicated. The figure shows the results obtained over two days of experimentation (n wt= 8, n KDB = 9 runs).

Fig. 4. Glucose-induced cytosolic free ATP changes in wild type MIN6 and Wolframin-depleted cells. Cells were infected with adenovirus expressing cytosolic luciferase and, 48 h later, perifused in the presence of 5 μmol/l luciferin in a photon-counting device as described under Materials and Methods. Representative traces for **A:** wild type MIN6 cells; **B** KDA; **C**: KDB. The y axes represent photon counts, proportional to cytosolic ATP concentration ($[ATP]_{CYT}$) (22,23). Note the reduced ATP rise in response to 30 mmol/l glucose in WFS1-depleted cells. **D** Quantitative analysis of the glucose induced rise in cytosolic ATP in wild type MIN6 and WFS1-depleted cells. The percentage increase in apparent free cytosolic ATP concentration in response to elevated glucose was about three-fold lower in KDA than in wild type MIN6 (***: wild type vs KDA: p=7.43 x 10^{-5}) and about ten-fold lower in KDB than in wild type MIN6 (***: wild type vs KDB: p= 1.8 x10^{-13}); n, wild type =12; n, KDA=6 and n, KDB=12 runs).

Fig. 5. SERCA expression in WFS1 depleted cell models. Representative immunoblots and bar charts showing quantitative analysis. **A:** SERCA expression in *WFS1* depleted MIN6 cells - microsomal fractions. Immunoblot: lane 1control (C), lane 2 KDB, 3 KDA. Top panel- PanSERCA antibody Y1F4 (32) used for detection, bottom panel- PDI antibody used for normalisation. 22µg of microsomal fraction extract was loaded per lane. (*: C vs KDA p=0.045, C vs KDB p=0.052, n= 6 runs with two independently prepared microsomal fraction extracts). **B:** SERCA expression in primary islets isolated from *Wfs1* conditional KO mice- whole protein extracts. (**: C vs KO p=0.005, n=4, using two independently prepared extracts). Top panel -PanSERCA antibody Y1F4 (32), bottom panel: beta actin (BA). 10µg of protein extract was loaded per lane. **C:** SERCA expression in *WFS1* depleted neuroblastoma SK-N-AS cells, total protein extracts. Top panel- Pan SERCA antibody Y1F4 (32), bottom panel beta actin (BA). (*: C vs KD1: p=0.02, n=3; **: C vs KD2: p=0.004, n=8; **: C vs KD3: p=0.009, n=8).Two independently prepared KD1 extracts and , five independently prepared KD2 and KD3 extracts were run. 17 µg of protein extract was loaded per lane. Lanes: 1 - control, 2 - KD1, 3 - KD2, 4 - KD3. **D:** Expression of SERCA2 isoform in *WFS1* depleted neuroblastoma SK-N-AS cells- total protein extracts. Top panel: isoform SERCA2 specific antibody (Santa Cruz), bottom panel Beta actin (BA). (*: C vs KD1: p=0.04, n=4; C vs KD2: p=0.02, n=6; C vs KD3: p=0.02, n=6). Two independently prepared KD1 extracts and 3 independently prepared KD2 and KD3 extracts were run. 17 µg of protein extract was loaded per lane. Lanes: 1-control, 2 -KD1, 3- KD2, 4 -KD3.

Fig.6. Co- immunoprecipitation of WFS1 and SERCA2. A and B: co-immunoprecipitation of WFS1 and SERCA2 in over-expressed system in Cos7 cells co-transfected with plasmids Myc-WFS1 and FLAG-SERCA2 or the relevant empty vectors as controls. **A:** Right hand side panels -IP: FLAG (immunoprecipitation with mouse monoclonal anti FLAG antibody, immunoblotting with either c-myc antibody (rabbit polyclonal, Sigma, top panel), WFS1 antibody (rabbit polyclonal, middle panel) or FLAG (rabbit polyclonal, bottom panel). Panels at the left hand side show the input: the expression of indicated proteins in extracts before co-immunoprecipitation (1% of extracts used in CO-IP). Lanes (on both: IP and input): 1: Myc-WFS1/ FLAG-SERCA2; 2: Myc-WFS1/empty-FLAG; 3: empty-

Myc/FLAG-SERCA2; 4: empty-Myc /empty-FLAG; lane 5 (on Co-IP gel): Input- extract expressing myc-WFS1 and FLAG-SERCA2. Myc-W1= Myc-WFS1, FL-SERC=FLAG-SERCA2, E-Myc= empty vector Myc, E-FL=empty vector FLAG. **B:** Right hand side panels: IP:Myc - immunoprecipitation with c- myc antibody (rabbit polyclonal,). Top panel: immunoblot with FLAG antibody (mouse monoclonal,), bottom panel immunoblot with c-myc (mouse monoclonal). Panels at the left hand side show the input: the expression of indicated proteins in extracts before co-immunoprecipitation (1% of extracts used in Co-IP). Lanes (on both : IP gel and input): 1 Myc-WFS1/ FLAG-SERCA2; 2: Myc-WFS1/empty- FLAG; 3: empty-Myc /FLAG-SERCA2; 4: empty-MYC /empty- FLAG. **C:** Co-IP of endogenous WFS1 and SERCA2 from SK-N-AS cells with anti WFS1 antibodies (rabbit polyclonal). Immunoblot: SERCA2 (goat polyclonal). Lanes 1-3: Input (1% of extracts used for Co-IPs in lanes 4- 6), lane 4: negative control: IP with anti FLAG antibody (rabbit polyclonal), lane 5: IP with WFS1 antibody (rabbit polyclonal), lane 6: negative control: IP with FLAG antibody (mouse monoclonal), n=4. **D:** Co-IP of endogenous WFS1 and SERCA2 from SK-N-AS cells after DTT treatment (n=3). Left panel: Input-expression of SERCA2 and WFS1 in DTT treated (D) and untreated (U) extracts before immunoprecipitation; note that there is no decrease in expression levels for neither of the proteins. Right panel: IP. Lanes: 1-input (2.5% of extract used for IP), -lanes 2 and 5 negative controls (IPs with FLAG antibody with untreated and treated extracts respectively); lane 3: IP with WFS1 antibody from DTT treated sample; lane 4: IP with WFS1 antibody from untreated sample. Top panel : immunoblotting with SERCA2 antibody, bottom panel: the same membrane probed with WFS1 antibody. Numbers below the gel panels show relative quantification of SERCA2 bands intensity normalized with WFS1.

Fig. 7. Proteasome inhibition results in increase of SERCA levels in WFS1 dependent manner. Control (C) and WFS1 depleted (KD2) neuroblastoma (SK-N-AS) cells were grown for 24 hours in 6 well plates and then treated with MG132 proteasome inhibitor (5μmol/l or 10μmol/l) for 4 hours before being harvested for protein analysis. The control samples were treated with equal volume of DMSO. The expression of SERCA was measured by Western blotting using PanSERCA antibody Y1F4 (32) and normalised to beta actin (BA). 11μg of protein extract was loaded per lane. Lanes (both

panels):1 DMSO: treatment, 2: MG132 at 5µmol/l, 3: MG132 at 10µmol/l. Bar chart- quantification of SERCA expression: (*: C v KD2 at 5µmol/l MG132: p= 0.01; C v KD2 at 10µm/l MG132: p= 0.03; control n=6 runs, KD2 n=4 runs using two independently treated extracts). MG132_5: treatment with 5µmol/l MG132; MG132_10: MG132 at 10µmol/l.

Fig 8. Regulation of ER calcium homeostasis by WFS1 via regulation of SERCA2 levels:

Under conditions of calcium homeostasis (normal conditions) WFS1 interacts with SERCA and negatively regulates SERCA turnover to maintain physiological SERCA2 levels. Under conditions of ER stress or in WFS1 depleted cells WFS1-SERCA2 interaction is limited: the complex is either not formed or formed at a reduced level. This results in SERCA2 upregulation. SERCA2 upregulation provides a compensatory mechanism which allows for increased Ca^{2+} pumping to the ER in response to Ca^{2+} leak from the ER caused by either ER stress or WFS1 depletion. This compensatory mechanism allows restoration of calcium homeostasis and prevention of cell death.

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Fig.1A

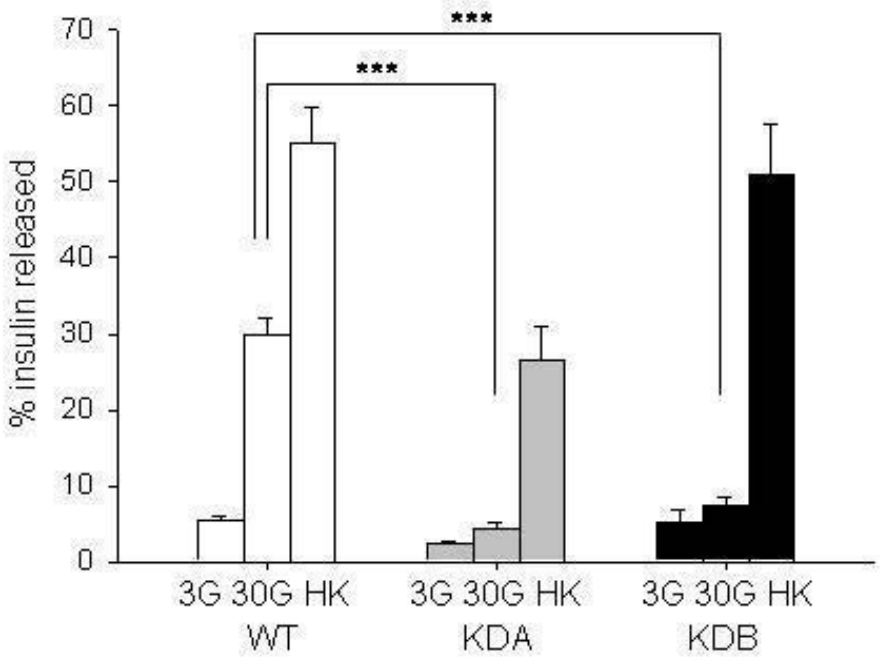
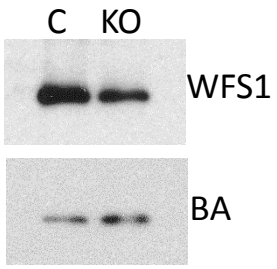
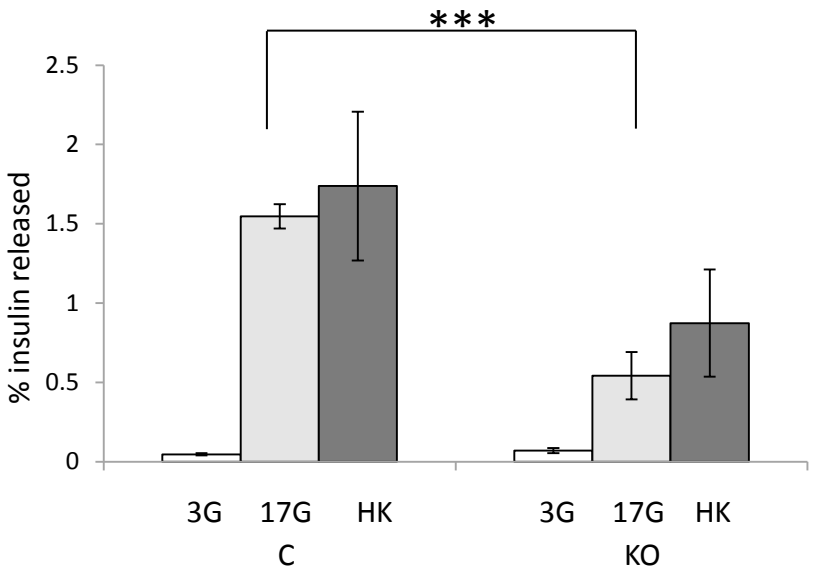
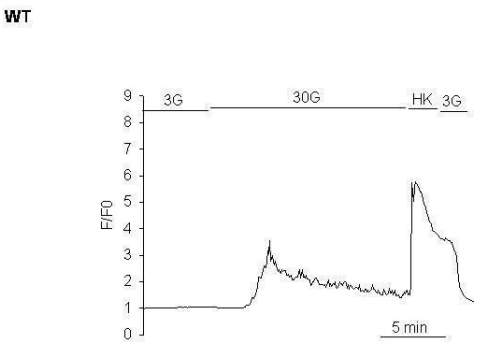

***
***
70
60
50
40
30
20
10
0
% insulin released
3G 30G HK
WT
3G 30G HK
KDA
3G 30G HK
KDB

Fig1B.

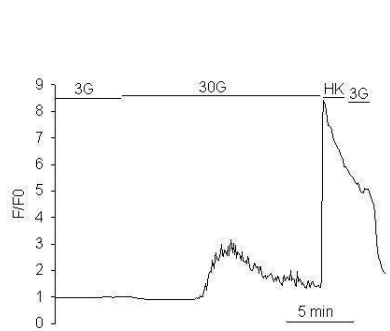

C
KO
WFS1
BA


***
2.5
2
1.5
1
0.5
0
% insulin released
3G
17G
HK
3G
17G
HK
C
KO

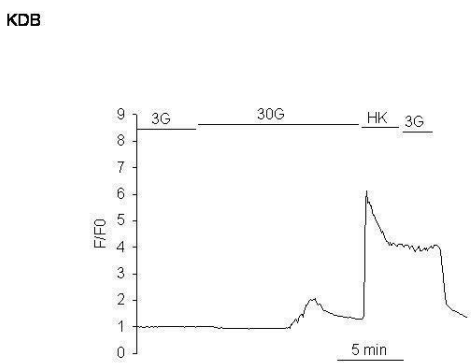
2A


WT
3G
30G
HK
3G
F/F0
5 min

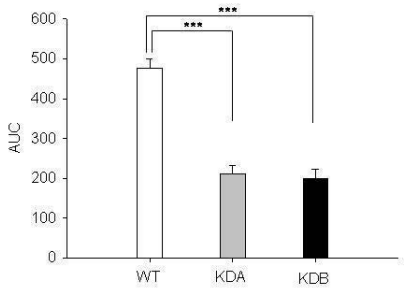
2B


KDA
3G
30G
HK
3G
F/F0
5 min

2C


KDB
3G
30G
HK
3G
F/F0
5 min

2D


AUC
***
***
WT
KDA
KDB

2E

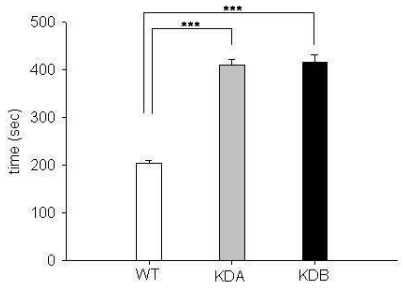

time (sec)
***
***
WT
KDA
KDB

Fig.3

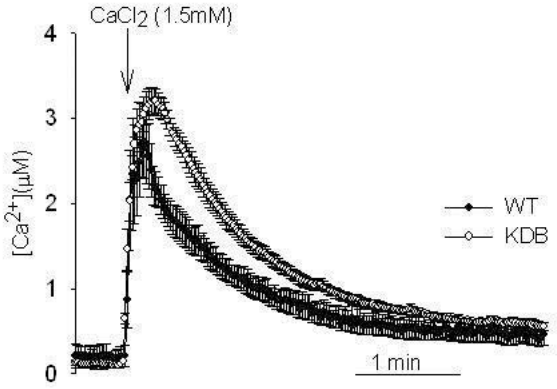


Fig.4A

wt

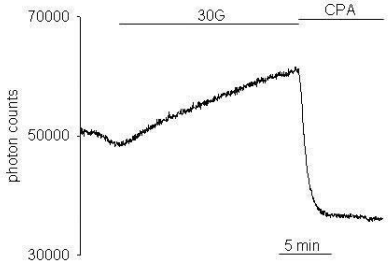


Fig.4B

KDA

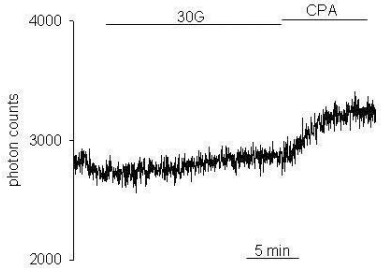


Fig.4C

KDB

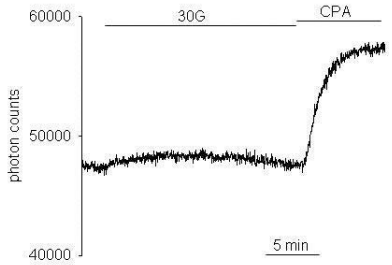
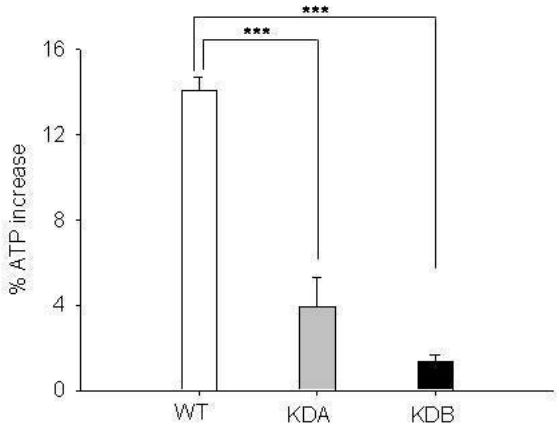
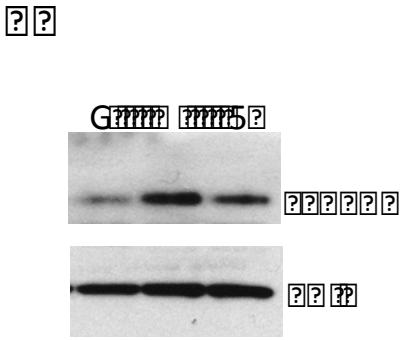
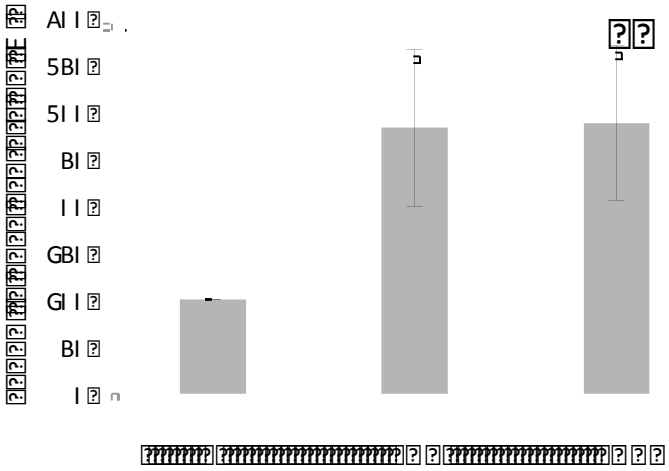
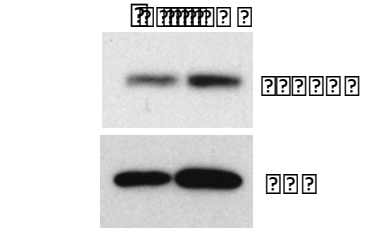


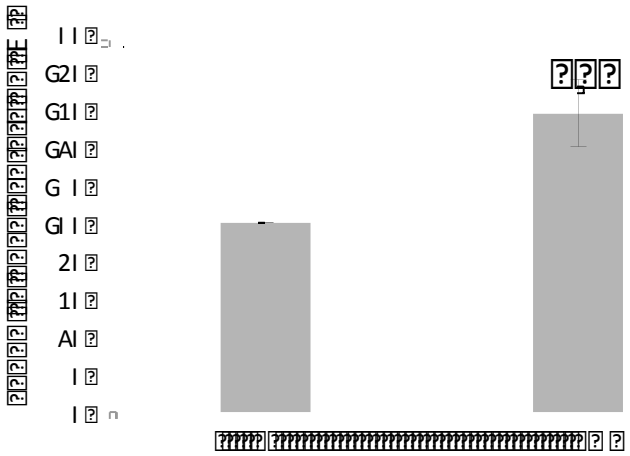
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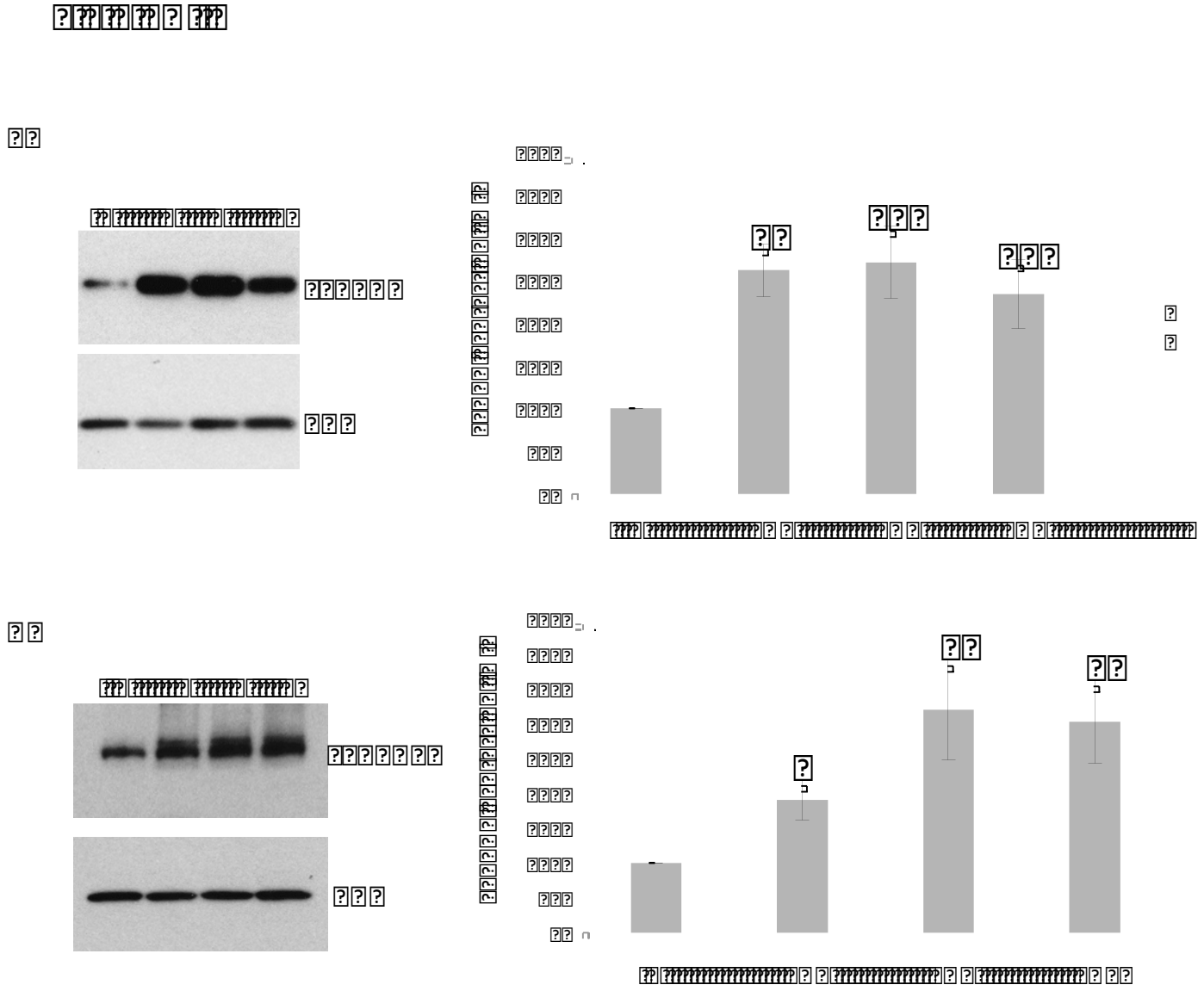












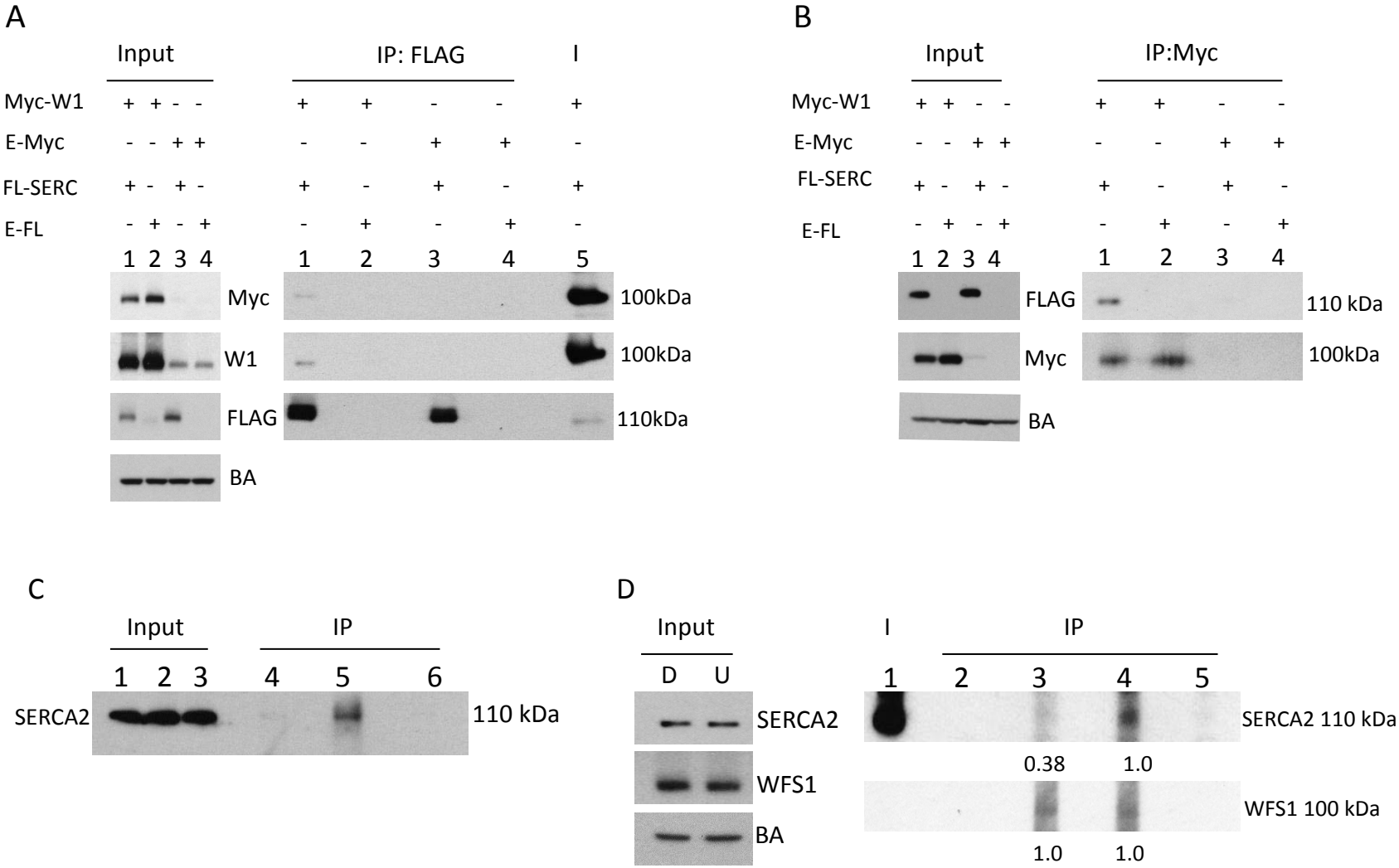
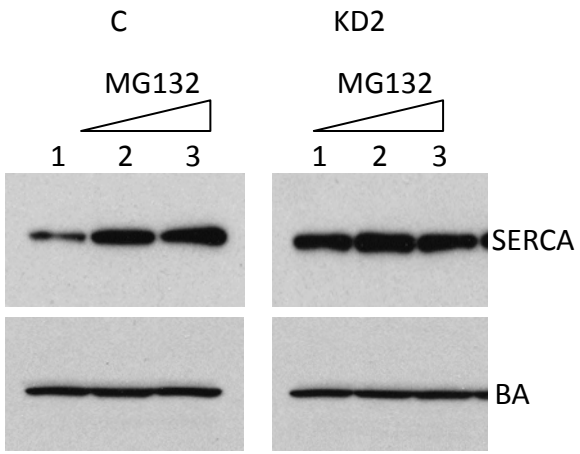
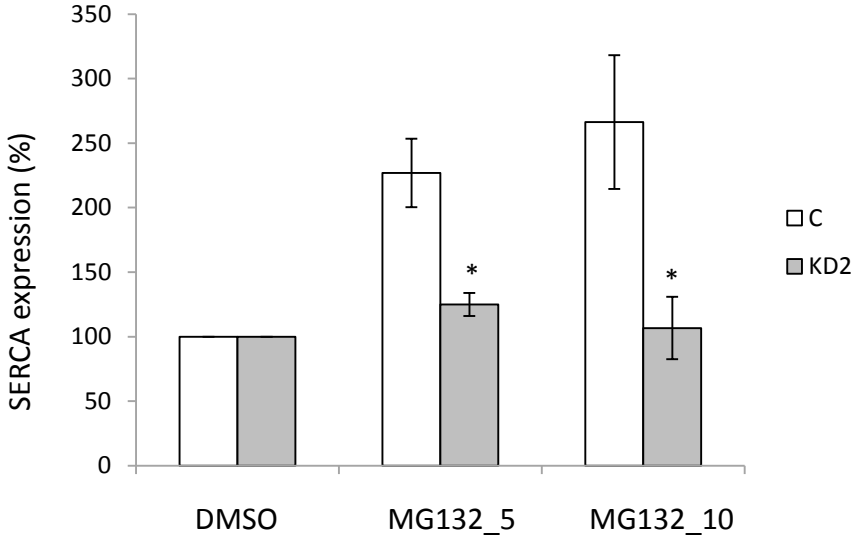
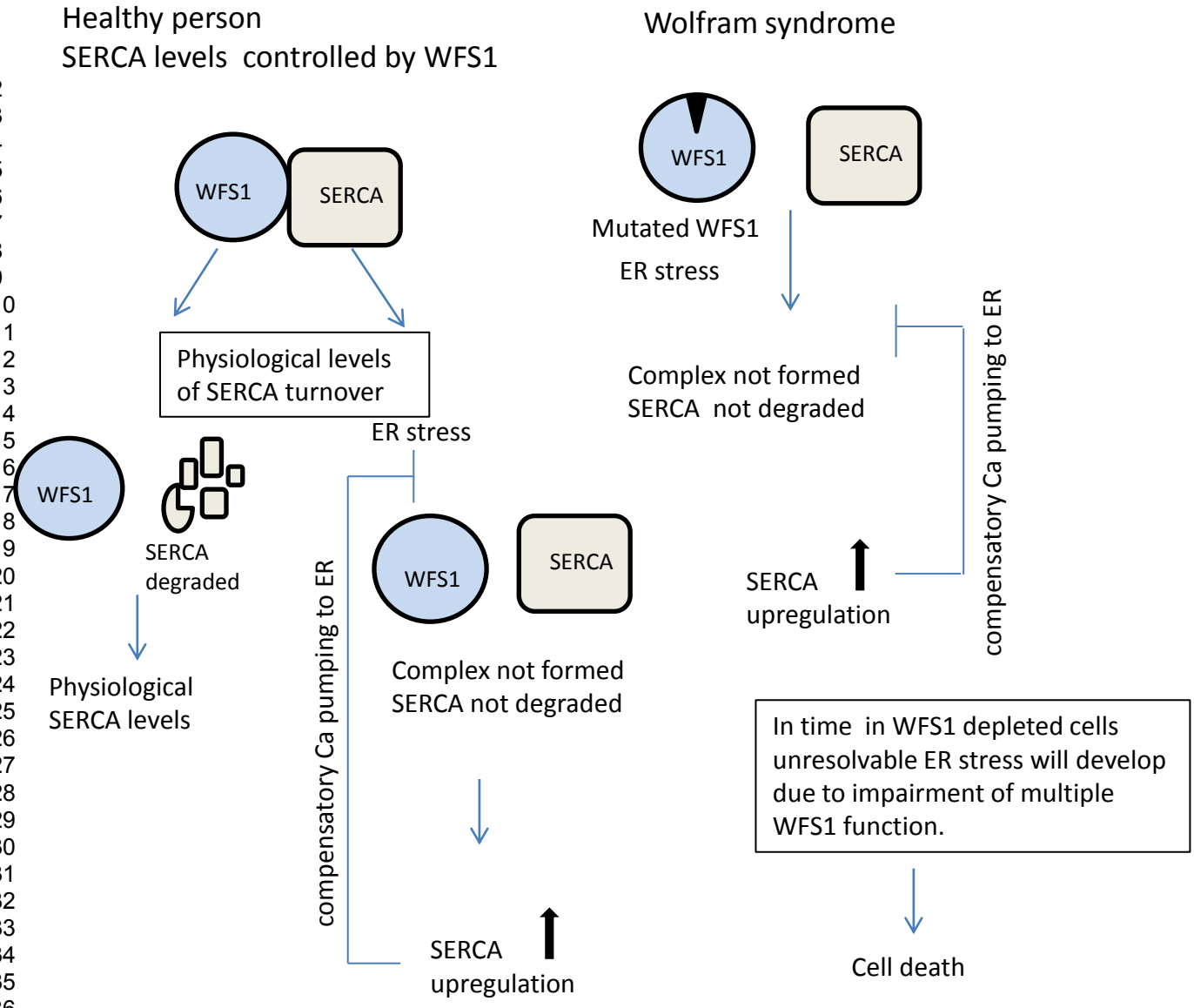

A
Input
IP: FLAG
I
Myc-W1
E-Myc
FL-SERC
E-FL
1 2 3 4
1 2 3 4 5
Myc
W1
FLAG
BA
100kDa
100kDa
110kDa
B
Input
IP:Myc
Myc-W1
E-Myc
FL-SERC
E-FL
1 2 3 4
1 2 3 4
FLAG
Myc
BA
110 kDa
100kDa
C
Input
IP
1 2 3 4 5 6
SERCA2
110 kDa
D
Input
D U
SERCA2
WFS1
BA
I
IP
1 2 3 4 5
SERCA2 110 kDa
0.38 1.0
WFS1 100 kDa
1.0 1.0

Fig 7.


C
KD2
MG132
MG132
1 2 3
1 2 3
SERCA
BA


SERCA expression (%)
350
300
250
200
150
100
50
0
DMSO
MG132_5
MG132_10
C
KD2
*
*


Healthy person
SERCA levels controlled by WFS1
WFS1
SERCA
Physiological levels of SERCA turnover
WFS1
SERCA degraded
Physiological SERCA levels
ER stress
WFS1
SERCA
Complex not formed SERCA not degraded
compensatory Ca pumping to ER
SERCA upregulation
Wolfram syndrome
WFS1
SERCA
Mutated WFS1
ER stress
Complex not formed SERCA not degraded
SERCA upregulation
compensatory Ca pumping to ER
In time in WFS1 depleted cells unresolvable ER stress will develop due to impairment of multiple WFS1 function.
Cell death