**Page 1 of 48 Human Molecular Genetics**

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2 **Sarco(endo)plasmic reticulum ATPase (SERCA) is a molecular partner of Wolfram**

3

4 **syndrome 1 (WFS1) protein, which negatively regulates its expression.**

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**Human Molecular Genetics**

**Page 2 of 48**

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2 **SUMMARY**

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4 **Wolfram syndrome is an autosomal recessive disorder characterised by neurodegeneration and**

5

6 **diabetes mellitus. The gene responsible for the syndrome (*WFS1*) encodes an endoplasmic**

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8 **reticulum (ER)-resident transmembrane protein that is involved in the regulation of the**

9

10 **unfolded protein response, intracellular ion homeostasis, cyclic AMP production and regulation**

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12 **of insulin biosynthesis and secretion. In this study, single cell Ca2+ imaging with fura-2 and**

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14 **direct measurements of free cytosolic ATP concentration ([ATP]CYT) with adenovirally**

15

16 **expressed luciferase confirmed a reduced and delayed rise in cytosolic free Ca2+ concentration**

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18 **([Ca2+]CYT), and additionally, diminished [ATP]CYT rises in response to elevated glucose**

19

20 **concentrations in WFS1 depleted MIN6 cells. We also observed that SERCA expression was**

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22 **elevated in several WFS1 depleted cell models and primary islets. We demonstrated a novel**

23

24 **interaction between WFS1 and SERCA by co-immunoprecipitation in Cos7 cells and with**

25

26 **endogenous proteins in human neuroblastoma cells. This interaction was reduced when cells**

27

28 **were treated with the ER stress inducer dithiothreitol (DTT). Treatment of WFS1 depleted**

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30 **neuroblastoma cells with the proteosome inhibitor MG132 resulted in reduced accumulation of**

31

32 **SERCA levels compared with wild type cells. Together these results reveal a role for WFS1 in**

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34 **the negative regulation of SERCA and provide further insights into the function of WFS1 in**

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36 **calcium homeostasis.**

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42 Word count 204

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**Page 3 of 48 Human Molecular Genetics**

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2 **INTRODUCTION**

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4 There is increasing evidence that endoplasmic reticulum (ER) perturbation plays a critical role in cell

5

6 death in both neurodegenerative disorders (1,2) and diabetes mellitus (3,4). Abnormal release of

7

8 calcium from the ER has been observed in several pathological conditions affecting the nervous

9

10 system (5,6). ER stress, oxidative stress, palmitate, and chronic high glucose, all decrease pancreatic

11

12 beta cell ER calcium levels, leading to beta cell death (7).

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14

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16 The ER functions as a calcium store through the expression of at least 3 types of proteins: the sarco-

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18 endoplasmic reticulum calcium ATPase (SERCA) family of proteins that actively pump calcium into

19

20 the ER; luminal calcium binding proteins for storing calcium; and the gated calcium channels inositol

21

22 trisphosphate receptors (IP3R) and ryanodine receptors (RyR) for the controlled release of calcium

23

24 from the ER along its electrochemical gradient (8). ER calcium depletion may be associated with,

25

26 among others, toxin interaction with the IP3R (GM1 gangliosidosis (9)); over-activation of the RyR

27

28 (Gaucher disease (6)); SERCA inhibition (Sandhoff disease (10)) and increased SERCA expression as

29

30 a compensatory mechanism through regulation by ATF6 during the ER stress response (11).

31

32

33

34 Childhood-onset diabetes mellitus and progressive optic atrophy are the diagnostic features of

35

36 Wolfram syndrome, a genetic form of both diabetes and neuro-degeneration (12). In this disease,

37

38 pancreatic beta cells and presumably neuronal cells are selectively destroyed due to mutations in the

39

40 *WFS1* gene, which encodes WFS1 protein or Wolframin, an ER transmembrane protein (13). The

41

42 mechanism is thought to be through perturbed ER homeostasis (14, 15) leading to ER stress (16,17).

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44 Loss of function mutations in *WFS1* have been shown to lead to ER calcium depletion (18), increased

**Human Molecular Genetics**

**Page 4 of 48**

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2 depleted MIN6 cells, we observed increased SERCA protein expression. These observations prompted

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4 us to investigate the possibility of an interaction between WFS1 protein and SERCA.

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**Page 5 of 48 Human Molecular Genetics**

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2 **RESULTS**

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4 **Wolframin-depleted beta cell lines show reduced insulin secretion in response to elevated glucose**

5

6 **or KCl-concentrations.**

7

8 Wolframin has been implicated in the normal control of stimulus-secretion coupling during regulated

9

10 insulin secretion *in vivo* and *in vitro* (16,17). To study the effects of Wolframin depletion on insulin

11

12 secretion and Ca2+ signalling in β-cells, we used the MIN6 insulinoma cell line. These stable cell lines

13

14 have been engineered to provide reduced *WFS1* expression using RNA interference, with 0% (wild

15

16 type), 50% (KDA) or 70% (KDB) reduction in Wolframin expression achieved by expression of

17

18 suitable scrambled or anti-*WFS1* shRNAs (16, 20).

19

20

21

22 Insulin release in response to glucose (30 vs 3 mmol/l) was decreased in *WFS1* depleted cells (Fig.

23

24 1A). At 30mmol/l glucose (30G) the percentage of insulin released was decreased seven-fold for

25

26 KDA (p=2.2 x10-7) and four-fold for KDB (p= 4.5 x 10-7) cells respectively in comparison to wild

27

28 type. At 3mmol/l glucose (3G), and after depolarisation with 50mmol/l KCl (HK), the percentage of

29

30 insulin released was also decreased in KDA and KDB cells, but only reached significance in the KDA

31

32 cells.

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37 **Wolframin-depleted primary islets from *WFS1* conditional KO mice show defective insulin**

38

39 **secretion in response to glucose.**

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42 To confirm that Wolframin is necessary for normal insulin secretion in response to high glucose we

43

44 isolated pancreatic islets from beta cell selective conditional *WFS1* knock out (KO) mice, a kind gift

**Human Molecular Genetics**

**Page 6 of 48**

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4 The isolation of islets and insulin secretion assay were performed as described in Materials and

5

6

7 Methods. The percentage of insulin released in response to glucose (17 vs 3mmol/l) during static

8

9 incubation for 1 h was decreased about 65% in islets from *Wfs1* KO mice in comparison to size

10

11 matched control islets: percentage of insulin released by control islets (C) = 1.55±0.08%, while by

12

13 KO islets= 0.54±0.15%, p=0.0006, (Fig1B). After depolarization with 20mmol/l KCl (HK) the

14

15 percentage of insulin released from KO islets was also reduced in comparison to the control but did

16

17 not reach statistical significance. These results confirmed the results obtained in MIN6 cells and are

18

19 consistent with previous *in vivo and* the *in vitro* findings (16,17, 21).

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23

24 **Wolframin depletion results in a delayed and reduced glucose-induced rise in cytoplasmic free**

25

26 **Ca2+.**

27

28 The above results suggested that alterations in glucose sensing may result in diminished glucose-

29

30 induced insulin secretion in *WFS1*-depleted cells. To explore this possibility further, we investigated

31

32 glucose- and KCl-induced cytosolic calcium ([Ca2+]i) rises in single, fura-2-loaded cells. The baseline

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34 [Ca2+]i for each cell type was calculated as the average of the first 180 seconds of each experiment in

35

36 perifusion buffer containing 3 mmol/l glucose, using the ratio of the emissions after exciting the dye

37

38 at 340 and 380nm. There were no apparent differences in baseline [Ca2+]i between the cell lines

39

40 (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

41

42 at the start of experiment was assumed to be equal 1for each cell line).

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**Page 7 of 48 Human Molecular Genetics**

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2 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,

3

4 KDB n=21 cells).

5

6

7

8 The delay before a detectable increase in [Ca2+]i following stimulation with 30 mmol/l glucose was

9

10 also more than 2-fold longer in *WFS1* depleted cells than in wild type cells (Fig. 2E). Whilst the wild

11

12 type cells responded after 203.7± 6.8 s, KDA and KDB cells responded only after 410.0 ± 12.9 and

13

14 416.7± 15.4 s respectively (*p* values: wild type vs KDA: p=6.2 x 10-18; wild type vs KDB: p=7.3 x

15

16 10-16; KDA v KDB: p=0.3). All cell lines responded similarly to depolarisation with 50 mmol/l KCl

17

18 (Fig. 2A, B, C). The response to 50mmol/l KCl was quantitated by measurement of the area under the

19

20 curve (AUC) and is presented on Fig. 2F (AUC for wild type, KDA and KDB were: 435.1±9.0;

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22 447.3± 15.8, 445.1± 16.2 respectively, wild type n=61, KDA n=36, KDB n=21 cells).

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24

25

26 **Cytosolic free Ca2+ concentrations at baseline are not significantly different in Wolframin**

27

28 **depleted cells compared to wild type cells.**

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30 To confirm that there was no apparent differences in baseline [Ca2+]i between the cell lines we

31

32 measured [Ca2+]CYT in wild type and KDB cells with cytosolic (untargeted) aequorin. Our results

33

34 (Fig.3) showed only a slightly higher level of cytosolic Ca2+ in KDB than in wild type cells. The

35

36 results come from two independent experiments (wild type n =8; KDB n =9 separate measurements),

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38 and are consistent with our earlier findings of no significant differences in baseline [Ca2+]i between the

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40 cell lines in single, fura-2-loaded cells.

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**Human Molecular Genetics**

**Page 8 of 48**

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2 (25). Representative traces showing the apparent increase in free [ATP]CYT (as photon counts) for wild

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4 type, KDA and KDB respectively are presented in Fig. 4 A,B,C, with quantitative analysis in Fig. 4D.

5

6 The increase in [ATP]CYT in response to high glucose was 14.1% ± 0.6 for the wild type MIN6 cells

7

8 (Fig. 4A), consistent with previous results (22,23,26) but only 4% ± 1.4 for KDA (p=7.43 x 10-5 )

9

10 (Fig. 4B) and 1.3% ± 0.3 for KDB (p= 1.8 x10-13) (Fig 4C). The apparent rise in [ATP]CYT in

11

12 response to high glucose was approximately three-fold less in KDA than in wild type MIN6 cells and

13

14 about ten-fold lower in KDB than in wild type MIN6 cells; (n, wt=12, n, KDA=6 and n, KDB=12 runs

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16 in three separate experiments for KDB and two separate experiments for KDA) (Fig. 4D).

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19

20 To determine whether changes in the apparent [ATP]CYT increase in response to glucose may be due to

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22 alterations in Ca2+ pumping into the ER or other intracellular stores, we monitored the impact of

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24 pharmacological depletion of these stores on the observed changes (Fig. 4). Interestingly, and in

25

26 contrast to previous studies (27), inhibition of SERCA pumps with cyclopiazonic acid (CPA)

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28 decreased the magnitude of the glucose-induced rise in [ATP]CYT in wild type cells, presumably

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30 reflecting enhanced ATP consumption for Ca2+ transport by other mechanisms (e.g. extrusion across

31

32 the plasma membrane by PMCA (plasma membrane Ca2+ ATPase) or transporting into the lumen of

33

34 Golgi with SPCA (secretory pathway Ca2+ ATPase) (Fig. 4A). By contrast, in both *WFS1* depleted

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36 cell lines, SERCA inhibition increased free [ATP]CYT (Fig 4B,C).

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39

40 To confirm the above results we investigated ATP:ADP ratio rise to glucose using a different (static)

41

42 method as described in Materials and Methods. Briefly, cells were grown in 6 well plates, starved

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44 overnight in medium with low glucose and on the day of the experiment incubated in buffers with

**Page 9 of 48 Human Molecular Genetics**

1

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

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4 cells.

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8 **Total ATP levels are reduced in WFS1 depleted cells.**

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10 We next measured total ATP content in wild type and *WFS1*-depleted MIN6 cells as described in

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12 Materials and Methods. The results were normalised for the cell number and presented as Relative

13

14 Luminescence Units (RLU, proportional to the amount of ATP) per 104 cells, (Table 1). The total

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16 levels of ATP (RLU) were significantly reduced in both WFS1 depleted cell lines to 79% (of the wild

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18 type) in KDA and 53% in KDB.

19

20

21

22 **SERCA expression is increased in WFS1 depleted cells.**

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25 The decreased levels of cytosolic ATP could have been the result of an ATP generation defect or ATP

26

27 overconsumption in WFS1 depleted cells. As no defect in oxidative phosphorylation was identified

28

29 previously in Wolfram patient biopsies (29), there was a possibility that ATP may be over-consumed

30

31 in WFS1 depleted cells. One of the major ATP consumers in pancreatic cells is the SERCA calcium

32

33 pump. We hypothesised that SERCA over-activity could result in ATP depletion in WFS1 depleted

34

35 cells and investigated the levels of SERCA expression.

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37

38 We examined SERCA expression levels in several WFS1 depleted cell models: MIN6 pancreatic

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40 cells, primary pancreatic islets isolated from conditional *Wfs1* KO mice and in WFS1 depleted SK-N-

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42 AS neuroblastoma cells (Fig 5). All the WFS1 depleted models displayed Unfolded Protein Response

43

44 (UPR) activity, measured as an increase in the levels of ER stress markers (16, 30).

**Human Molecular Genetics**

**Page 10 of 48**

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2 To examine the SERCA expression levels in isolated islets we prepared total protein extracts from 10-

3

4 13 weeks old *Wfs1* conditional KO males as described in Materials and Methods. There was a greater

5

6 than 50% increase of SERCA levels in *Wfs1* conditional KO islets in comparison to *Wfs1* positive

7

8 controls: (C=100%, KO=157.8±17.8, p=0.005, n=4 Fig 5B). Wolfram syndrome manifests also as a

9

10 neurodegenerative disease. Therefore we used three WFS1-depleted neuroblastoma cell lines (KD1-

11

12 KD3) depleted by 60-80% in comparison to the control (C) as described by Gharanei et al., (30) and

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14 examined the SERCA expression using either Pan-SERCA antibody Y1F4 (Fig 5C) or isoform

15

16 SERCA2 specific antibody (Fig 5D). The expression levels of SERCA were increased in all WFS1-

17

18 depleted cell lines. Fig 5C shows the increased expression levels detected with Pan SERCA antibody

19

20 (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,

21

22 n=8, C=100%). We confirmed that this increase is mainly due to an increase in SERCA2 expression.

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24 Fig 5D shows increased levels of isoform SERCA2 (KD1=189.8±28.6%, p=0.04, n=4;

25

26 KD2=318.9±71.2%, p=0.02, n=6; KD3=301.7±59.3%, p=0.02, n=6, C=100%).

27

28

29 In summary, all WFS-depleted models showed increased levels of SERCA expression, (the main

30

31 contributor being isoform SERCA2). This is consistent with our hypothesis that in WFS1-depleted

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33 cells SERCA may be overactive.

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38 **WFS1 interacts with SERCA2.**

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41 To explain how the absence of WFS1 protein may affect the levels of SERCA expression we

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43 hypothesized that the two proteins may be molecular partners. We first examined the potential WFS1-

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45 SERCA2 interaction in an over-expression system. We co-transfected FLAG-SERCA2 plasmid

**Page 11 of 48 Human Molecular Genetics**

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2 show that with anti FLAG antibody we were able to precipitate Myc-WFS1 (lane1). No WFS1 was

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4 detected in lanes 2-4, where either Myc-WFS1/empty FLAG (lane2), empty Myc /FLAG-SERCA2

5

6 (lane3) or empty Myc /empty FLAG (lane 4) plasmids were present. We probed the membrane with

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8 polyclonal rabbit anti FLAG antibody (bottom panel) to show the input.

9

10

11 In a reciprocal experiment (Fig 6B, right hand side panel) polyclonal rabbit anti c-myc antibody was

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13 used for co-immunoprecipitation and monoclonal mouse anti FLAG antibody for detection (top

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15 panel). Only in lane1, where both Myc-WFS1 and FLAG-SERCA2 proteins were expressed, we

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17 observed co-immunoprecipitation of FLAG-SERCA2. There was no SERCA2 detected in lanes 2-4,

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19 where either Myc-WFS1/empty FLAG (lane2), empty Myc/ FLAG-SERCA2 (lane3) or empty Myc

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21 /empty FLAG (lane4) were present. The bottom panel probed with monoclonal mouse c-myc

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23 antibody shows the input. These results indicate that WFS1 and SERCA2 can interact in an over-

24

25 expression system.

26

27

28 Next we examined if endogenous WFS1 and SERCA2 can be co-immunoprecipitated from SK-N-AS

29

30 neuroblastoma cells (Fig 6C). WFS1 antibody (rabbit polyclonal) were used for precipitation (lane5)

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32 and a ~110 kDa band was detected with SERCA2 antibody. No SERCA2 was detected in either lane

33

34 4 or 6 where negative controls: either FLAG- rabbit polyclonal or FLAG -mouse monoclonal antibody

35

36 were used for co-immunoprecipitation. Our results indicate that the two proteins, WFS1 and SERCA2

37

38 may interact with each other in SK-N-AS neuroblastoma cells.

39

40

41 To understand the role of this interaction we examined if WFS1 and SERCA2 can interact with each

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43 other under conditions of ER stress. We prepared protein extracts from neuroblastoma SK-N-AS cells

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45 either untreated or treated with ER stress inducer: 1mmol/l DTT for 3 hours. First, we examined the

**Human Molecular Genetics**

**Page 12 of 48**

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2 untreated extract was used. Interestingly, the SERCA2 band in the DTT treated sample was much

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4 reduced (Fig 6D, lane3, top panel). The membrane was re-probed with sheep WFS1antibody and the

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6 amount of precipitated WFS1 quantified showing equal amounts in both DTT treated and untreated

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8 samples (DTT:1.0, Untreated:1.0, Fig.6D, lower panel). Next, the amount of precipitated SERCA2

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10 was quantified, and normalized for the WFS1 levels: (untreated sample: SERCA2 =1.0; DTT treated

11

12 sample: SERCA=0.38). In summary, DTT treatment did not affect either the protein expression or the

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14 amounts of precipitated WFS1 (Fig 6D). This suggests that the 62% reduced levels of the amount of

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16 precipitated SERCA2 in DTT-treated sample do not result from reduced levels of precipitated WFS1

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18 under these conditions. Our results thus suggest that WFS1-SERCA2 interaction may be impaired

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20 under ER stress conditions.

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

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29 We demonstrated above that WFS1 interacts with SERCA and SERCA expression is increased in

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31 WFS1 depleted cells. We hypothesized that WFS1 may be a negative regulator of SERCA and targets

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33 SERCA to proteasome mediated degradation. To test our hypothesis we compared SERCA levels in

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35 control and the WFS1 depleted cell line KD2 after treatment with the proteasome inhibitor MG132.

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37 Control and WFS1 depleted KD2 cells were treated with either 5 or 10µmol/l MG132 proteasome

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39 inhibitor as described by Gharanei et al., (30). Equal amounts of protein (11µg) was loaded per lane

40

41 and samples were resolved on SDS PAGE gels. SERCA expression in DMSO treated sample was

42

43 assumed to be equal 100%. Quantification revealed that while in control cells SERCA expression at

44

45 5µmol/l increased to 226.9± 26.5% and at 10µmol/l increase to 266.3±51.9% (2.2 and 2.6 fold

**Page 13 of 48 Human Molecular Genetics**

1

2 degradation. However, the ubiquitnation assay, in which we compared SERCA ubiquitnation levels

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4 between KD2 and control cells did not show impaired SERCA ubiquitination in KD2.

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9 **DISCUSSION**

10

11 In Wolfram syndrome, diabetes mellitus results from multiple defects in the glucose signalling

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13 pathway; and both the diabetes mellitus and neurodegeneration are thought to be associated with ER

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15 calcium depletion. The present study provides additional insights into its role in both mouse pancreatic

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17 beta cell and human neuroblastoma cell models of the disease. We observed the following: (1) our

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19 models confirmed that WFS1 protein depletion in MIN6 beta cells and primary islets resulted in

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21 reduced glucose stimulated insulin secretion, and reduced and delayed cytosolic Ca2+ rise to glucose;

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23 (2) we observed a reduced cytosolic ATP rise in response to high glucose, and reduced total cellular

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25 ATP content; (3) SERCA expression levels were increased in WFS1 protein depleted MIN6 cells,

26

27 WFS1 KO primary islets, and WFS1 protein depleted human neuroblastoma cells; (4) WFS1 protein

28

29 interacted with SERCA in both overexpressed and endogenous models; (5) Proteasomal inhibition

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31 studies suggested a role for WFS1 in SERCA protein turnover. (6) When ER stress was induced with

32

33 DTT, there was a reduced WFS1/SERCA complex formation or partial dissociation, allowing SERCA

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35 expression to increase. These results are consistent with a model in which WFS1 protein negatively

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37 regulates the ER stress response and modulates ER calcium filling by regulating SERCA expression to

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39 partially compensate for ER calcium depletion in conditions of ER stress.

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43 We showed that in two MIN6 β-cell models of Wolframin depletion there was reduced glucose

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45 stimulated insulin secretion (Fig 1A). We also demonstrated the defect in glucose stimulated insulin

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47 secretion in primary islets isolated from conditional beta cell specific knock out mice (Fig.1B). In our

48

49 MIN6 model of Wolframin depletion, this was associated with a delayed and reduced amplitude of

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the [Ca2+]i rise (Fig 2), and reduced [ATP]CYT rise in response to elevated glucose (Fig 4). These

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**Human Molecular Genetics**

**Page 14 of 48**

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2 Our findings of reduced [ATP]CYT rise in response to elevated glucose, and reduced total cellular ATP,

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4 suggest reduced ATP synthesis or increased ATP consumption after WFS1 silencing (22,27). This

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6 reduced [ATP]CYT rise may explain the observed reduced and delayed glucose-induced [Ca2+]i rise in

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8 these mutants (due to failed closure of ATP-sensitive K+ channels). ATP generation defects have not

9

10 been previously reported in Wolfram syndrome; our own previous work demonstrated normal

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12 oxidative phosphorylation in muscle biopsies from Wolfram patients (29). Increased ATP

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14 consumption could arise from cellular efforts to restore ER homeostasis. SERCA pump activity has

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16 been shown to be a major ATP-consuming process in MIN6 cells (27) and has also been proposed as a

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18 negative regulator of [ATP]CYT, contributing to the Ca2+-dependent oscillations in [ATP]CYT observed

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20 in beta cells (33,34).

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24 These observations led us to hypothesise that ATP utilisation by SERCA may increase in the absence

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26 of WFS1 protein; and further, that increased activity/expression of SERCA would be observed in

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28 WFS1 protein depletion. To test our hypothesis we studied the levels of SERCA expression in several

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30 models of WFS1 depleted cells: pancreatic MIN6, SK-N-AS neuroblastoma and in primary islets

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32 isolated from *WFS1* conditional knock out mice. All our models showed elevated ER stress markers

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34 (16,30). We found that SERCA levels were significantly elevated in all our models and that it was

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36 mainly due to increased levels of isoform SERCA2 (Fig 5). This is a novel finding and to the best of

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38 our knowledge, there are no published reports of SERCA expression in WFS1 protein depleted cell

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40 models.

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43

44 We demonstrated that WFS1 and SERCA interact in both an over-expression system (Fig 6AB) and

45

46 endogenously (Fig 6C); and that this interaction is reduced in the presence of DTT-induced ER stress

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49 (Fig 6D). Inhibition of the proteasome with MG132 resulted in accumulation of SERCA in a WFS1

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51 dependent manner: the accumulation of SERCA expression was reduced in WFS1 depleted

**Page 15 of 48 Human Molecular Genetics**

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2 depleted cells (data not shown). We tested for “canonical “ signals for proteasomal recognition with

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4 antibody against ubiquitin lysine-48 based chains. However, several “non canonical” ubiquitin based

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6 signals for proteasomal targeting have also been described (e.g. polyubiquitin chains assembled

7

8 through residues other than lysine 48), tagging substrates with “ubiquitin like” proteins, or

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10 proteasomal degradation without prior ubiquitination (35). Therefore the mechanism of WFS1

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12 regulation of SERCA requires further confirmation.

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16 It seems that the regulation of SERCA protein levels by WFS1 may be ER stress dependent: under

17

18 conditions of ER stress SERCA is released from the interaction with WFS1 (or alternatively SERCA-

19

20 WFS1 complexes are formed at a lower rate), it may be degraded by the proteasome to a lesser extent,

21

22 and SERCA expression increases. Under normal conditions of ER homeostasis the physiological

23

24 levels of SERCA may be maintained in homeostasis by interaction with WFS1. A similar function for

25

26 WFS1 was described by Fonseca et al., (15) who demonstrated that WFS1 negatively regulates a key

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28 transcription factor involved in ER stress signalling Activating Transcription Factor 6α (ATF6α)

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30 through the ubiquitin-proteasome pathway.

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34 Some forms of ER stress result in ER calcium depletion, disturbed ER functions and increase in

35

36 cytosolic calcium levels which may trigger cell death via activation of the calpain-2 apoptotic pathway

37

38 (7). We provide evidence that under conditions of WFS1 protein depletion, there may be a secondary

39

40 increase in SERCA expression to pump calcium ions back into the ER calcium store to try to restore

41

42 ER homeostasis. Several reports show that induction of ER stress may lead to increased SERCA

43

44 expression on both mRNA and protein levels (36,37,38,11,39). In addition Wu et al., (40) reported that

45

46 cytosolic calcium elevation itself increased SERCA2 expression by a mechanism distinct from ER

47

48

49 stress. The authors speculated that enhanced calcium uptake into the ER might shorten the period of a

50

51 relative ER calcium depletion subsequent to a stimulus–induced ER calcium release, in this way

**Human Molecular Genetics**

**Page 16 of 48**

1

2 function may increase to restore ER calcium levels; in irresolvable ER stress, ER calcium levels

3

4 cannot be restored SERCA expression falls and apoptosis ensues.

5

6

7

8 The absence of WFS1 is associated with ER stress (14,15,16,17), calcium leak from the ER and an

9

10 elevation of cytosolic calcium levels (7). ER stress, ER calcium leak and elevations of cytosolic

11

12 calcium have all been associated with increases in SERCA expression (11,36,37,38,40). ATF6 is also

13

14 negatively regulated by WFS1, and a downstream effect of increased ATF6 activity is elevation of

15

16 SERCA expression (11). It may be that under these conditions for the cells to survive SERCA

17

18 expression must be increased to counteract the ER calcium leak to restore ER calcium homeostasis

19

20 and to prevent activation of cell death pathway induced by elevated cytosolic calcium levels. The

21

22 decrease of SERCA levels under ER stress and under some pathological conditions observed by Hara

23

24 et al., (7) was in the presence of WFS1, where induction of this compensatory mechanism was not

25

26 necessary; WFS1 exerted its pro-survival function and prevented cell death. Our results, summarised

27

28 on Fig 8, are consistent with the possibility that WFS1 negatively regulates SERCA turnover, possibly

29

30 via proteasome mediated degradation and this process is dependent on the ER stress. Release of

31

32 SERCA from interaction with WFS1 in WFS1 depleted cells or under ER stress may result in

33

34 increased SERCA levels and activity and may allow compensatory pumping of calcium ions from

35

36 cytosol to ER lumen to restore ER calcium homeostasis and prevent cell death.

37

38

39

40 To further understand WFS1 role in calcium homeostasis it will be interesting to examine if/how

41

42 WFS1 depletion affects the expression of inositol trisphosphate (IP3) receptors and ryanodine

43

44 receptors (RYR), the calcium channels that release calcium from the ER; and how WFS1 depletion

45

46 affects other intracellular Ca2+ stores.

47

48

49

50

51 In summary, our results support previously reported WFS1 involvement in regulation of glucose

**Page 17 of 48 Human Molecular Genetics**

1

2 **Materials and Methods.**

3

4 **Cell culture and adenoviral infection**

5

6 Mouse β-cell-derived MIN6 cells expressing interfering RNAs to provide reduction in *WFS1*

7

8 expression (16) were cultured in Dulbecco’s modified Eagle’s medium (Sigma) containing 25mmol/l

9

10 glucose, 2mmol/l pyruvate, supplemented with 15% (vol/vol) FBS, 4mmol/l glutamine, 100 units/ml

11

12 penicillin, 100 µg/ml streptomycin, 143 µmol/l β-mecaptoethanol and 0.2 mg/ml geneticin. Luciferase

13

14 expression was achieved by infection of cells with adenoviral vector encoding cytoplasmic-targeted

15

16 luciferase (AdCMVcLuc) (22), aequorin expression by using untargeted cytosolic aequorin (Cyt.Aq)

17

18 (43). Human neuroblastoma SK-N-AS cells with stable WFS1 depletion were grown as described by

19

20 Gharanei et al., (30).

21

22

23

24 **Insulin secretion from MIN6 cells.**

25

26 Cells were seeded in six-well plates and grown in full growth medium for at least 16 hours, before

27

28 they were maintained overnight in medium with 3mmol/l glucose. Insulin secretion was performed as

29

30 previously described [(http://www.jbc.org/content/280/27/25565.long](http://www.jbc.org/content/280/27/25565.long)) and insulin content measured by

31

32 radioimmunoassay (Millipore).

33

34

35

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

37

38

39 The mice used in this experiment were beta cells selective conditional *Wfs1* knock-out mice (16),

40

41 obtained by collaboration from Professor A. Permutt. They were additionally back crossed with C57

42

43 wild type females for two generations and the heterozygotes were used as parents in matings to obtain

44

45 experimental and control littermates. Islets were isolated by collagenase digestion from pancreata of

46

47 10-13 weeks old male mice: either *Wfs1* conditional knock out mice (*Wfs1* fl/fl *Cre* cre/+ ) or *Wfs1*

48

49 positive “floxed” littermates (*Wfs1* fl/fl ). Islets of Langerhans were isolated, cultured and insulin

50

51 secretion assay performed as previously described (26,44).

**Human Molecular Genetics**

**Page 18 of 48**

1

2 µl of buffer). For homogenization the lysate was multiple times frozen (at -80C) and defrosted at RT,

3

4 each time being passed several times through pipette tip followed by passing through a 21.5G size

5

6 needle (26,44).

7

8

9

10

11 **Cytosolic free Ca2+ concentration measurements with fura-2**

12

13

14 Cells were seeded on 24 mm diameter coverslips and starved in medium containing 3mmol/l glucose

15

16 overnight. The next day, cells were loaded with the fluorescent dye fura-2 (5µmol/l), and incubated in

17

18 Krebs buffer (see above) with 3mmol/l glucose. Next, the cells were perifused with buffer containing

19

20 3mM glucose followed by 30mmol/l glucose, then 50mmol/l KCl as a control. Changes in ([Ca2+]i) in

21

22 single cells were measured as the changes in fluorescence intensity of fura-2 using an Olympus IX-80

23

24

inverted optics epifluorescence microscope (x40 oil immersion objective). Single cell [Ca2+]

i

25

26 measurements were performed exciting the dye at 340 and 380nm and emission was recorded at

27

28

29 510nm. Images were recorded with an IMAGO charge-coupled device camera (Till Photonics GmbH,

30

31 Grafelfing, Germany) controlled by Tillvision software (Till Photonics).

32

33

34

35 **Measurements of cytosolic free Ca2+ with recombinant targeted aequorin.**

36

37 Cells were seeded onto 13 mm diameter coverslips and 24 h later, at 70-80% confluency, transfected

38

39 with adenoviruses expressing the untargeted (cytosolic) bioluminescent protein aequorin (45,46).

40

41 Forty eight hours later, cells were depleted of Ca2+ by incubation with 10µmol/l ionomycin, 10µmol/l

42

43 cyclopiazonic acid (CPA) and 10µmol/l monensin in modified Krebs-Ringer bicarbonate buffer

44

45 containing glucose 3mmol/l, Hepes 10mmol/l, KCl 3.5mmol/l, MgSO4 0.5mmol/l, NaCl 140mmol/l,

46

47 NaHCO3 2mmol/l, and NaH2PO4 0.5mmol/l (to achieve pH 7.4), and supplemented with EGTA

48

49 1mmol/l for 5 min at 4C (46). Cytosolic free calcium levels were measured using a cytoplasmic (non

50

51 targeted) aequorin construct and native coelenterazine (47). Aequorin was reconstituted with 5µmol/l

**Page 19 of 48 Human Molecular Genetics**

1

2 to the perifusion chamber and converted to Ca2+ concentration [µmol/l] using algorithms described

3

4 previously (50,51).

5

6

7

8 **Cytosol free ATP concentration measurements**

9

10 Cells were seeded onto 13 mm diameter coverslips and 24h later, at 70-80% confluency infected with

11

12 adenovirus expressing untargeted (cytosolic) firefly luciferase (22,23). After 48h culture, the cells

13

14 were “starved” overnight in medium containing 3mmol/l glucose. The following day, the cells were

15

16 incubated for a further 15 min. in modified Krebs buffer containing 3mmol/l glucose, then perifused in

17

18 the same buffer containing 3mmol/l glucose, followed by buffer with 30mmol/l glucose and then

19

20 followed by addition of CPA (10µmol/l), an inhibitor of SERCA. The cytosolic free ATP

21

22 concentration ([ATP]CYT) was estimated by counting the emitted photons with a photomultiplier as

23

24 described above.

25

26

27

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

29

30 Cells were seeded in 6 well plates in standard media. The next day the cells were starved overnight in

31

32 the medium with 3mmol/l glucose. On the day of experiment the cells were incubated for 15 min in

33

34 Krebs buffer with 3mmol/l glucose; and after that they were incubated for 15 min in Krebs buffer

35

36 with either 3mmol/l glucose (plate1), 30mmol/l glucose (plate2) or 30mmol/l glucose and CPA

37

38 (plate3). The plates 1 and 2 were harvested after 15 min in 200µl per well of 20% (v/v) ice cold

39

40

41 perchloric acid (PCA) and rapidly frozen in -80°C. 10µmol/l CPA was added to plate 3 and incubated

42

43 additional 5 min before being harvested in PCA as above. In parallel, another plate was seeded in

44

45 identical way for measurements of protein concentration to normalise the results and harvested in

46

47 protein lysis buffer (RIPA). Standard curve for ATP and ADP (a negative control) was prepared

48

49 (gradient of concentrations versus luminescence). The samples (harvested in PCA) were neutralised to

50

51 pH7.4 with a known volume of neutralisation mixture (0.5mol/l triethanolamine, 2mol/l KOH and

**Human Molecular Genetics**

**Page 20 of 48**

1

2 where ADP was converted to ATP by addition of pyruvate kinase. After two hours incubation at RT,

3

4 10µg of luciferase and 1mmol/l luciferin were added to each sample and luminescence was measured

5

6 on luminometer for both: set1 (ATP) and set2 (ATP+ADP). The amount of ADP was calculated as a

7

8 difference ADP = (ATP+ADP)-ATP (28).

9

10

11

12

13

14 **Total cellular ATP content**

15

16 Cells were grown to 90% confluency in standard medium (see above) with 25mmol/l glucose,

17

18 harvested, diluted 10x in growing medium, counted and seeded in opaque-walled 96 wells plates

19

20 (Appleton Woods). The following day total ATP assay was performed using the CellTiter-Glo

21

22 Luminescent Cell Viability Assay (Promega). Briefly, the plates were equilibrated to room

23

24 temperature for 30 min, and a volume of reconstituted CellTiter-Glo Reagent (100µl to 100µl of

25

26 medium in each well) was added. The content was mixed for 2 min. in an orbital shaker to induce

27

28 lysis; after 10 min incubation in room temperature the luminescence was read on a multilabel counter

29

30 Wallac 1420 Victor3 (Perkin Elmer).

31

32

33

34 **Preparation of microsomal fractions.**

35

36

37 8 x T75cm flasks of each: wt, KDA and KDB MIN6 (80% confluent) were harvested by

38

39 trypsinisation, rinsed with PBS and resuspended in 2mls of PBS on ice. Cells were spun down at 2000

40

41 rpm, 5 min, 4ºC and suspended in 10mls of MEMBRANE buffer (5mM HEPES, 0.32M sucrose pH7.2

42

43 with protease inhibitors) before they were homogenised using electric homogeniser Polytron, ultra

44

45 Turro T8 at 30 000rpm for 10 sec. Then, cells were transferred to glass electric homogeniser and

46

47 homogenised with 10 strokes. Cells were spun down at 10000g, 10min, 4ºC to remove organelles

48

49 (nuclei and mitochondria). Cloudy supernatant (15mls) was harvested to a separate tube, divided

50

51 between two ultracentrifuge tubes (3/4 full) and spun down at 100 000x g for 1 hour at 4˚C.

**Page 21 of 48 Human Molecular Genetics**

1

2 **Proteasome inhibition assay.**

3

4 Cells were plated at 5 × 105 cells/well in a 6-well plate. After 24h, cells were treated with either 5 or

5

6 10µmol/l MG132 proteasome inhibitor for 4h, harvested in RIPA buffer and prepared for Western

7

8 Blotting as described by Gharanei et al., (30).

9

10

11

12

13 **Immunoblotting.**

14

15 Antibody were diluted in 5% milk in PBS-Tween and used at the following concentrations:

16

17 PanSERCA Y1F4 (mouse monoclonal (32)) 1:5000; SERCA2 (goat polyclonal Santa Cruz) 1:1000;

18

19 Beta actin (mouse monoclonal Sigma) 1:14000; PDI (mouse monoclonal Abcam) 1:5000. For protein

20

21 detection in extracts from isolated islets the following concentrations of primary antibody were used:

22

23 WFS1 (rabbit polyclonal, obtained by collaboration (20)) 1:500; PanSERCA Y1F4 1:500, beta actin

24

25 1:3500. Secondary antibody (anti rabbit, goat and mouse, Dako) were used at 1:20000.

26

27

28

29

30

31 **Co-immunoprecipitation.**

32

33

34 *Construction of FLAG-SERCA2 plasmid:* full length human SERCA2 (transcript variant b, GeneBank

35

36 NM\_170665.3) was amplified from a human cDNA library by PCR using primers 5’-

37

38 CTTGCGGCCGCGATGGAGAACGCGCAC-3’ (forward) and 5’-

39

40 GCATGGTACCTCAAGACCAGAAGATATCG-3’ (reverse) and was cloned between the Not I and

41

42 Kpn I site of pFLAG-CMV4 expression vector (Sigma). The sequence was confirmed by DNA

43

44 sequencing. pCMV-Myc-WFS1 plasmid was described before (20).

45

46

47

48 *Transfection:* Cos7 cells were seeded at 2.0 x 106 in 10cm plates and after 24h co-transfected with the

49

50 following combinations of plasmids (2µg of each): pCMV-Myc-WFS1/FLAG-SERCA2, pCMV-Myc-

51

52 WFS1/empty FLAG, empty Myc /FLAG-SERCA2 and empty Myc/empty FLAG using Turbofect

**Human Molecular Genetics**

**Page 22 of 48**

1

2 *Immunoprecipitation in over-expression system:* 750µg of protein extract and either anti-FLAG mouse

3

4 monoclonal antibody (Sigma) or anti c-myc rabbit, polyclonal antibody (Sigma) were used for co-

5

6 immunoprecipitation in the over-expression system. The extracts were incubated with antibody

7

8 overnight at 4°C with end to end rotation. Protein G Sepharose beads were added after overnight

9

10 incubation for a further 4 hours of end to end rotation at 4°C. Beads were separated from lysate using

11

12 Spin-X columns (Costar) by centrifugation at 4000 x g for 15 min at 4°C and washed 4 times with

13

14 RIPA buffer (for washes:1, 2 and 4 RIPA buffer with 150mmol/l NaCl was used, for wash 3 RIPA

15

16 buffer with NaCl increased to 500mmol/l was used). Each wash was performed by 15min incubation

17

18 at 4˚C with end to end rotation, followed by spin at 4000 x g. Bound proteins were eluted in 50µl

19

20 sample buffer by heating to 70°C for 8 min followed by 3min spin at a maximum speed in microfuge

21

22 and stored in -80°C. The following antibody were used for immunoblotting: c-myc (rabbit polyclonal,

23

24 Sigma) 1:10000; WFS1 (rabbit polyclonal, Proteintech) 1: 500; FLAG (rabbit polyclonal Sigma)

25

26 1:1000; FLAG (mouse monoclonal, Sigma) 1:1000; c-myc (mouse monoclonal Sigma) 1:1000.

27

28 Secondary anti mouse and anti rabbit antibody (Dako) were used 1:20000.

29

30

31

32 *DTT treatment*. 80% confluent SK-N-AS cells growing in 10cm dishes were treated with 1mmol/l

33

34 DTT (Sigma) for 3 hours or remained untreated before being harvested in 600µl TBD (composition

35

36 below). Equal amounts of untreated and DTT treated protein lysates were used for co-

37

38 immunoprecipitation as described below.

39

40

41

42 *Immunoprecipitation of endogenous proteins:* To detect endogenous interaction neuroblstoma SK-N-

43

44 AS cells in 10cm dishes were harvested in 600µl Tris buffer (TBD) per dish (20mmol/l Tris pH7.5,

45

46 with 100mmol/l NaCl, 1%TritonX-100, 1mmol/l DTT, 1mmol/l PMSF and protease inhibitors

47

48

49 (Complete Mini, Roche )), scraped on ice, placed in eppendorfs and incubated at 4˚C with end to end

50

51 rotation for 1 hour. The samples were sonicated 3x10s on ice and the lysates cleared by

52

53 centrifugation at 23000 x g at 4˚C. 900µg-1mg of protein extract (400µl) was used per reaction.

**Page 23 of 48 Human Molecular Genetics**

1

2 columns (Costar) with 20µl of pre-cleared Protein G Sepharose beads, for 3h incubation at 4˚C with

3

4 end to end rotation. The samples were centrifuged at 4000 x g at 4˚C and the beads washed 2x in

5

6 400µl of buffer TBD-150 (with 150mmol/l NaCl), 1x in TBD-500 (with 500mmol/l NaCl), and again

7

8 1x in TBD-150. Each time the beads were incubated 15-20 min at 4°C with end to end rotation.

9

10 Finally beads were separated from lysates by centrifugation at 4000xg and 50µl of SDS loading

11

12 buffer was added per sample. The proteins were released from beads by heating to 70˚C for 8 min,

13

14 spun down at 13000rpm in microfuge for 3 min and supernatant harvested. The samples were run on

15

16 SDS-PAGE gels, transferred to membranes at (90V for 90 min) and boiled for 4 minutes in water

17

18 before being blocked in 5% milk in PBS-Tween for 1 hour. The membranes were incubated with

19

20 SERCA2 goat polyclonal antibody (Santa Cruz) 1:1000 followed by anti goat secondary antibody

21

22 (Dako) 1:20000, or WFS1 sheep polyclonal antibody (R&D systems) 1: 10000 followed by secondary

23

24 anti sheep (R&D systems) 1:30000 overnight at 4˚C with rotation.

25

26

27

28 *Statistical analysis*

29

30

31 Data are given as the means ± S.E. for the number of experiments given. Comparisons between means

32

33 were performed using Student’s t-test with Bonferonni correction for multiple testing, as appropriate.

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**Human Molecular Genetics**

**Page 24 of 48**

1

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5

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7

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9

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11

12 kindly gave advice on co-immunoprecipitation conditions.

13

14

15

16 **Conflicts of interest**

17

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

19

20 declare.

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**Page 25 of 48 Human Molecular Genetics**

1

2 **TABLE AND LEGEND**

3

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

5

6 Total ATP content was assayed using CellTiter-Glo Luminescent Cell Viability Assay (Promega) as

7

8 described under Material and Methods. The results are presented as relative luciferase units (RLU) per

9

10 104 cells and as a per cent of ATP content in wild type. The results come from 3 independent

11

12 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

13

14 *p*=1.0 x 10 -8 ).

15

16

17

18

19

20 **Table 1**

21

22

23

24

25

26

|  |  |  |
| --- | --- | --- |
| **Cell line** | **RLU/104 cells ± SE** | **% (total ATP of wt)** |
| **pSuper (WT)** | **378691.2± 9283** | **100%** |
| **KDA** | **297797.4± 10111** | **78.6%** |
| **KDB** | **198801.5± 12976** | **52.5%** |

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**Human Molecular Genetics**

**Page 26 of 48**

1

2 **FIGURE LEGENDS**

3

4

5 **Fig. 1. Glucose and depolarisation-induced insulin secretion from MIN6 cells and isolated**

6

7 **pancreatic islets.**

8

9 **A.** Insulin secretion from wild type and WFS1-depleted MIN6 cells. Quantitative analysis of insulin

10

11 secreted in response to 30 mmol/l glucose and 50 mmol/l KCl. Total and released insulin were

12

13 measured by radioimmunoassay as given under Materials and Methods. Wolframin depletion resulted

14

15 in a reduction of the percentage of insulin released in response to 30 mmol/l glucose (\*\*\*: wt vs KDA

16

17 *p*=2.2 x10-7, and wt vs KDB *p*= 4.5 x10-7). The results were from three experiments performed in

18

19 triplicate. 3G: 3 mmol/l glucose, 30G: 30 mmol/l glucose, HK (high potassium): 50 mmol/l KCl.

20

21 **B**. Glucose induced insulin secretion from primary islets isolated from conditional, beta cell selective

22

23 *Wfs1* KO mice**.** Immunoblot – 70% reduced WFS1 expression in islets isolated from *Wfs1* KO mice

24

25 in comparison to the control islets; 10µg of protein extract was loaded per lane. BA: beta actin. Bar

26

27 chart: Quantitative analysis of insulin secreted in response to 17 mmol/l glucose and 20 mmol/l KCl

28

29 measured by radioimmunoassay. The percentage of insulin released in response to 17 mmol/l glucose

30

31 is significantly reduced in islets from KO mice in comparison to size matched control islets (C): (\*\*\*:

32

33 C vs KO p=0.0006). The percentage of insulin released in response to high potassium (20 mmol/l) is

34

35 also reduced in islets from KO mice, but it did not reach statistical significance (C vs KO p=0.22). No

36

37 difference was measured in response to 3mmol/l glucose (C vs KO p=0.21). The results come from at

38

39 least three experiments in duplicates (for control islets (C) N=3, for KO islets N=6). 3G: 3 mmol/l

40

41 glucose, 17G: 17 mmol/l glucose, HK (high potassium): 20 mmol/l KCl.

42

43

**Page 27 of 48 Human Molecular Genetics**

1

2 glucose, 30G: 30 mmol/l glucose, HK (high potassium): 50mmol/l KCl. **D.** Quantitative analysis of the

3

4 increase in free cytosolic Ca2+ concentration (fura-2) to 30 mmol/l glucose measured as AUC (area

5

6 under curve). The response in WFS1 depleted cells was more than 2-fold smaller than *wt* (\*\*\*: wild

7

8 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

9

10 response of cytosolic calcium rise to 30 mmol/l glucose in Wolframin-depleted cells. The delay before

11

12 a detectable response was more than 2.0 fold longer in WFS1 depleted cells than in wild type cells.

13

14 (\*\*\*: 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,

15

16 wild type=61; n, KDA=36; n, KDB=21 cells). **F.** Quantitative analysis of the increase in free

17

18 cytosolic calcium concentration (fura-2) to 50mmol/l KCl measured as AUC; there was no differences

19

20 between WFS1 depleted cells and WT MIN6.

21

22

23

24 **Fig. 3. Changes in cytosolic free calcium concentration in wild type MIN6 and Wolframin**

25

26 **depleted KDB cells.** Cells were infected with adenovirus expressing untargeted (cytosolic) aequorin.

27

28 48 h later, cells were depleted of calcium (see Materials and Methods), then perifused in nominally

29

30 Ca2+-free buffer, followed by buffer with 1.5mmol/l CaCl2 as indicated. The figure shows the results

31

32 obtained over two days of experimentation (n wt= 8, n KDB = 9 runs).

33

34

35

36 **Fig. 4. Glucose-induced cytosolic free ATP changes in wild type MIN6 and Wolframin-depleted**

37

38 **cells**. Cells were infected with adenovirus expressing cytosolic luciferase and, 48 h later, perifused in

39

40 the presence of 5 µmol/l luciferin in a photon-counting device as described under Materials and

41

42

43 Methods. Representative traces for **A:** wild type MIN6 cells; **B** KDA; **C**: KDB. The y axes represent

**Human Molecular Genetics**

**Page 28 of 48**

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3

4 **Fig. 5. SERCA expression in WFS1 depleted cell models**. Representative immunoblots and bar

5

6 charts showing quantitative analysis. **A:** SERCA expression in *WFS1* depleted MIN6 cells -

7

8 microsomal fractions. Immunoblot: lane 1control (C), lane 2 KDB, 3 KDA. Top panel- PanSERCA

9

10 antibody Y1F4 (32) used for detection, bottom panel- PDI antibody used for normalisation. 22µg of

11

12 microsomal fraction extract was loaded per lane. ( \*: C vs KDA p=0.045, C vs KDB p=0.052, n= 6

13

14 runs with two independently prepared microsomal fraction extracts). **B:** SERCA expression in

15

16 primary islets isolated from *Wfs1* conditional KO mice- whole protein extracts. (\*\*: C vs KO

17

18 p=0.005, n=4, using two independently prepared extracts). Top panel -PanSERCA antibody Y1F4

19

20 (32), bottom panel: beta actin (BA). 10µg of protein extract was loaded per lane. **C:** SERCA

21

22 expression in *WFS1* depleted neuroblastoma SK-N-AS cells, total protein extracts. Top panel- Pan

23

24 SERCA antibody Y1F4 (32), bottom panel beta actin (BA). ( \*: C vs KD1: p=0.02, n=3; \*\*: C vs

25

26 KD2: p=0.004, n=8; \*\*: C vs KD3: p=0.009, n=8).Two independently prepared KD1 extracts and ,

27

28 five independently prepared KD2 and KD3 extracts were run. 17 µg of protein extract was loaded per

29

30 lane. Lanes: 1 - control, 2 - KD1, 3 - KD2, 4 - KD3. **D:** Expression of SERCA2 isoform in *WFS1*

31

32 depleted neuroblastoma SK-N-AS cells- total protein extracts. Top panel: isoform SERCA2 specific

33

34 antibody (Santa Cruz), bottom panel Beta actin (BA). (\*: C vs KD1: p=0.04, n=4; C vs KD2: p=0.02,

35

36 n=6; C vs KD3: p=0.02, n=6). Two independently prepared KD1 extracts and 3 independently

37

38 prepared KD2 and KD3 extracts were run. 17 µg of protein extract was loaded per lane. Lanes: 1-

39

40 control, 2 -KD1, 3- KD2, 4 -KD3.

41

42

43

44 **Fig.6. Co- immunoprecipitation of WFS1 and SERCA2.** A and B: co-immunoprecipitation of

**Page 29 of 48 Human Molecular Genetics**

1

2 Myc/FLAG-SERCA2; 4: empty-Myc /empty-FLAG; lane 5 (on Co-IP gel): Input- extract expressing

3

4 myc-WFS1 and FLAG-SERCA2. Myc-W1= Myc-WFS1, FL-SERC=FLAG-SERCA2, E-Myc=

5

6 empty vector Myc, E-FL=empty vector FLAG. **B:** Right hand side panels: IP:Myc -

7

8 immunoprecipitation with c- myc antibody (rabbit polyclonal,). Top panel: immunoblot with FLAG

9

10 antibody (mouse monoclonal,), bottom panel immunoblot with c-myc (mouse monoclonal). Panels

11

12 at the left hand side show the input: the expression of indicated proteins in extracts before co-

13

14 immunoprecipitation (1% of extracts used in Co-IP). Lanes (on both : IP gel and input): 1 Myc-

15

16 WFS1/ FLAG-SERCA2; 2: Myc-WFS1/empty- FLAG; 3: empty-Myc /FLAG-SERCA2; 4: empty-

17

18 MYC /empty- FLAG. **C:** Co-IP of endogenous WFS1 and SERCA2 from SK-N-AS cells with anti

19

20 WFS1 antibodies (rabbit polyclonal). Immunoblot: SERCA2 (goat polyclonal). Lanes 1-3: Input (1%

21

22 of extracts used for Co-IPs in lanes 4- 6), lane 4: negative control: IP with anti FLAG antibody (rabbit

23

24 polyclonal), lane 5: IP with WFS1 antibody (rabbit polyclonal), lane 6: negative control: IP with

25

26 FLAG antibody ( mouse monoclonal), n=4. **D:** Co-IP of endogenous WFS1 and SERCA2 from SK-

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28 N-AS cells after DTT treatment (n=3). Left panel: Input-expression of SERCA2 and WFS1 in DTT

29

30 treated (D) and untreated (U) extracts before immunoprecipitation; note that there is no decrease in

31

32 expression levels for neither of the proteins. Right panel: IP. Lanes: 1-input (2.5% of extract used for

33

34 IP), -lanes 2 and 5 negative controls (IPs with FLAG antibody with untreated and treated extracts

35

36 respectively); lane 3: IP with WFS1 antibody from DTT treated sample; lane 4: IP with WFS1

37

38 antibody from untreated sample. Top panel : immunoblotting with SERCA2 antibody, bottom panel:

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40 the same membrane probed with WFS1 antibody. Numbers below the gel panels show relative

41

42 quantification of SERCA2 bands intensity normalized with WFS1.

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**Human Molecular Genetics**

**Page 30 of 48**

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2 panels):1 DMSO: treatment, 2: MG132 at 5µmol/l, 3: MG132 at 10µmol/l. Bar chart- quantification of

3

4 SERCA expression: (\*: C v KD2 at 5µmol/l MG132: p= 0.01; C v KD2 at 10µm/l MG132: p= 0.03;

5

6 control n=6 runs, KD2 n=4 runs using two independently treated extracts). MG132\_5: treatment with

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8 5µmol/l MG132; MG132\_10: MG132 at 10µmol/l.

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

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16 Under conditions of calcium homeostasis (normal conditions) WFS1 interacts with SERCA and

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18 negatively regulates SERCA turnover to maintain physiological SERCA2 levels. Under conditions of

19

20 ER stress or in WFS1 depleted cells WFS1-SERCA2 interaction is limited: the complex is either not

21

22 formed or formed at a reduced level. This results in SERCA2 upregulation. SERCA2 upregulation

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24 provides a compensatory mechanism which allows for increased Ca2+ pumping to the ER in response

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26 to Ca2+ leak from the ER caused by either ER stress or WFS1 depletion. This compensatory

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29 mechanism allows restoration of calcium homeostasis and prevention of cell death.

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**Page 31 of 48 Human Molecular Genetics**

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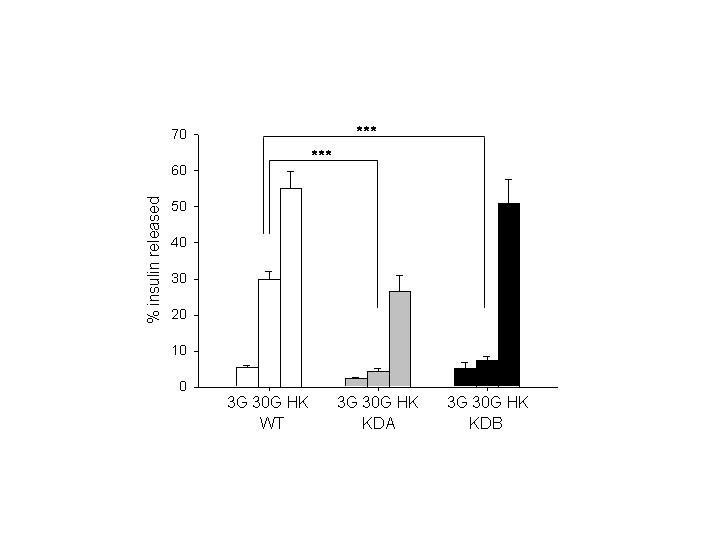
**Page 39 of 48 Human Molecular Genetics**

**For**

**Peer**

**Review**

Fig.1A



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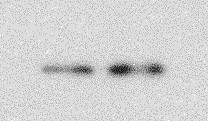
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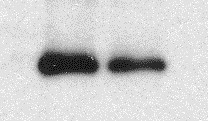


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**Human Molecular Genetics**

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**Page 40 of 48**

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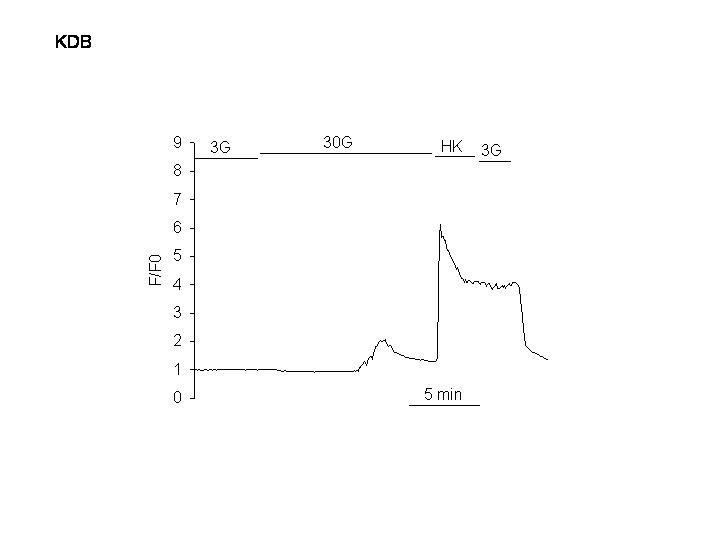
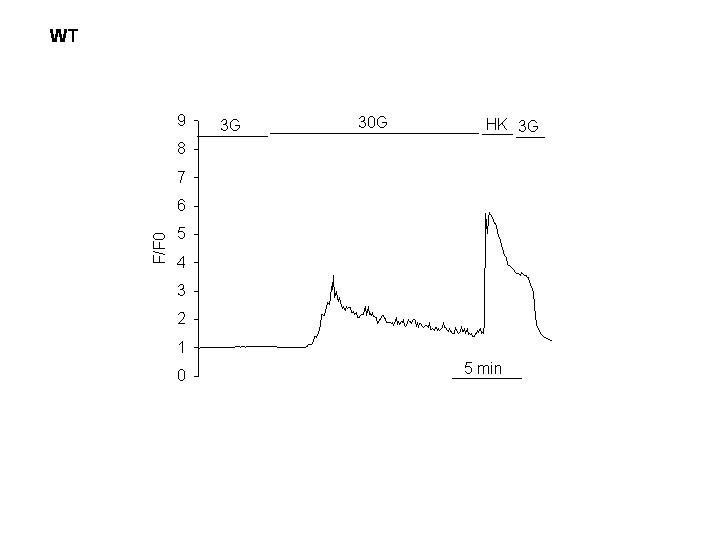
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**For**

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**Review**

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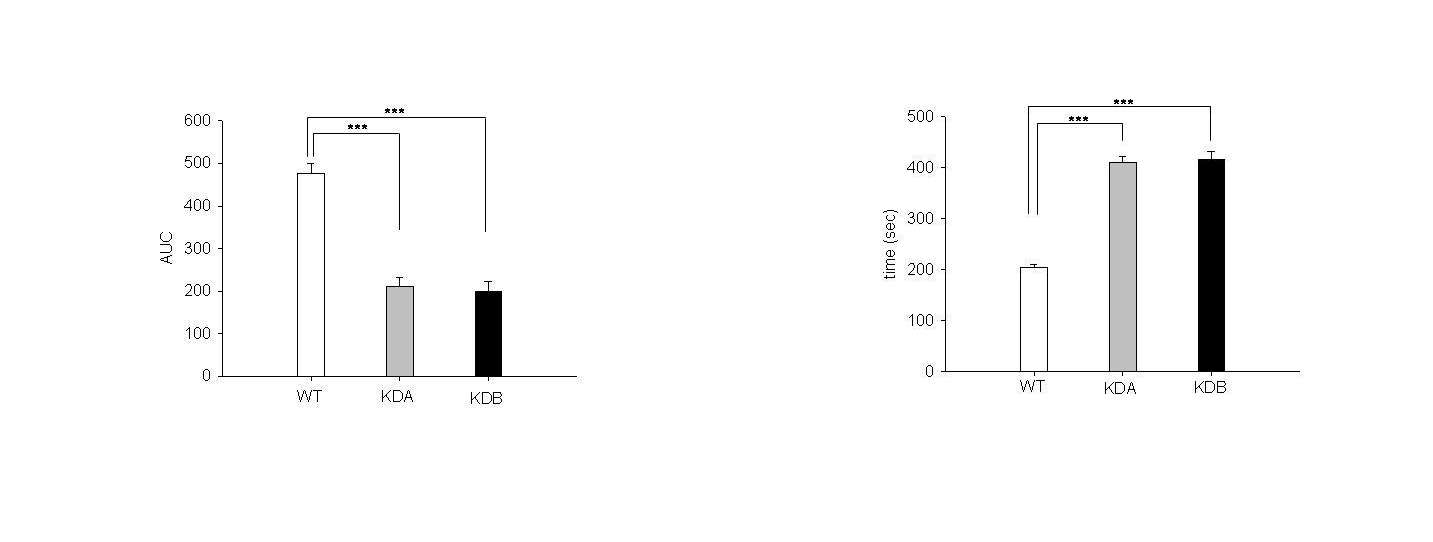
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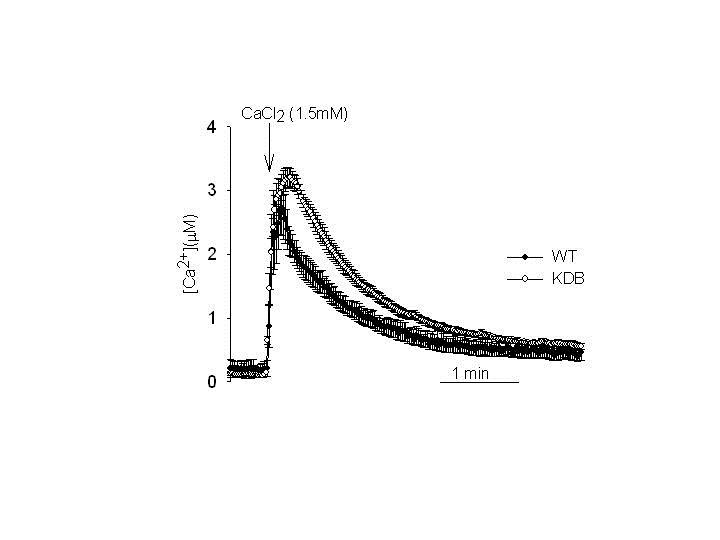
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**Human Molecular Genetics**



**Page 42 of 48**

**For**

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**Review**

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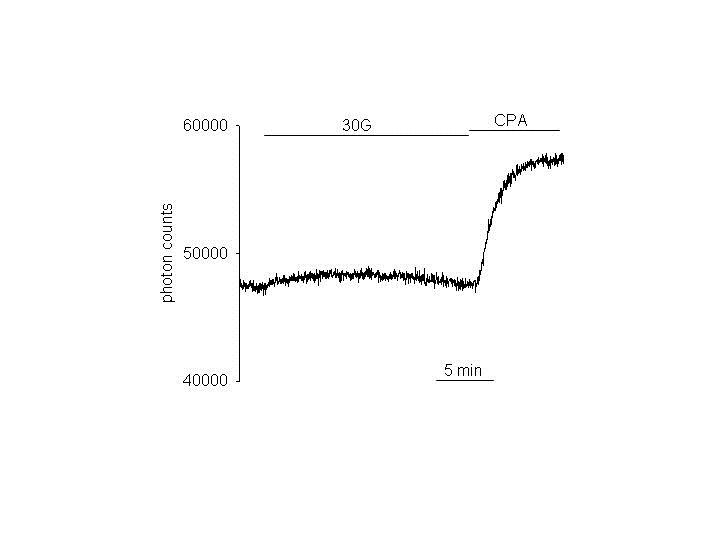
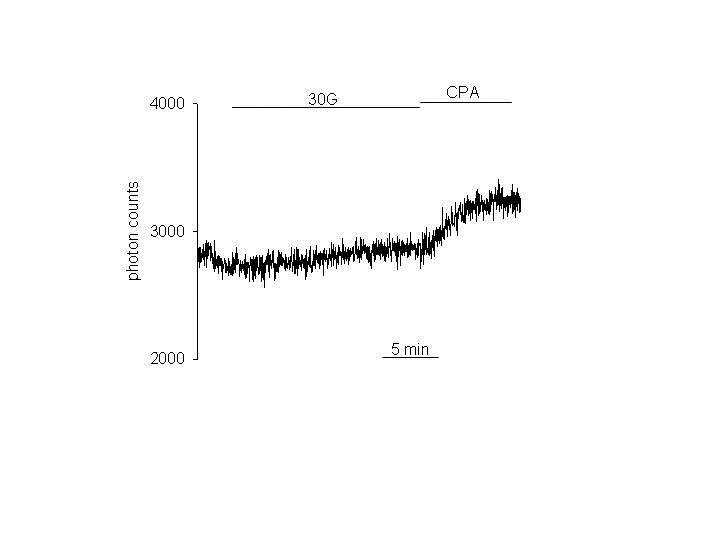
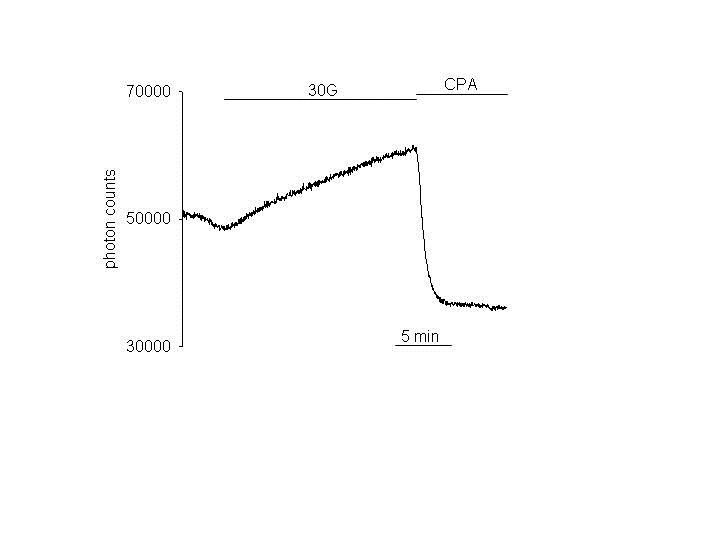
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**Page 43 of 48 Human Molecular Genetics**



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**Review**

Fig.4A Fig.4B Fig.4C

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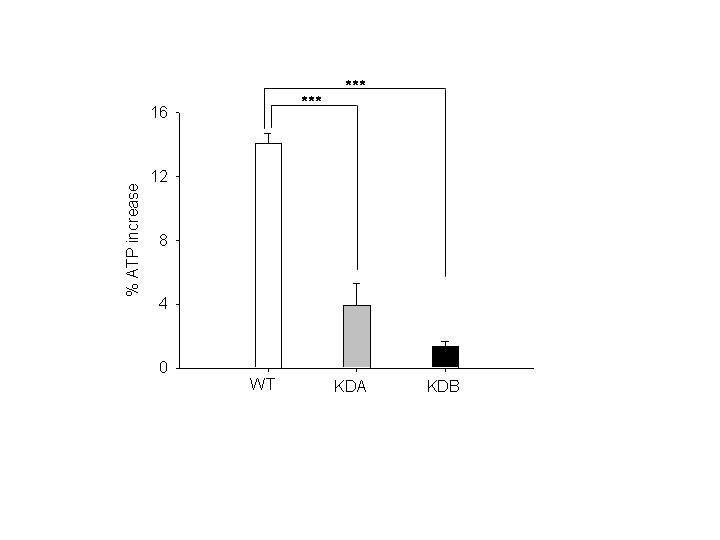
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**Human Molecular Genetics**

**Page 44 of 48**

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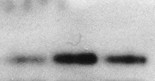
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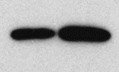
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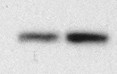
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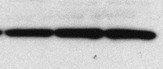
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SERCA  BA



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**Page 45 of 48 Human Molecular Genetics**

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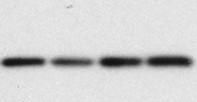
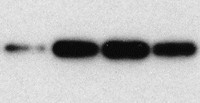
15

16

17

18

 1              2          3              4



SERCA  BA

SERCA  expression  (%)

400

350

300

250

200

SERCA2  expression  (%)

150

100

50

0

\*

\*\*

\*\*

19

**For**

**Peer**

**Review**

20

21

22

23 D

24

25

26

27

28

29

30

31

32

33

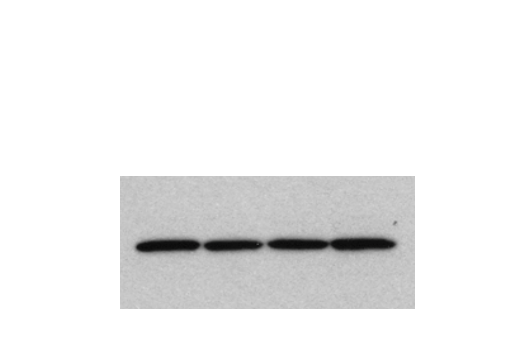
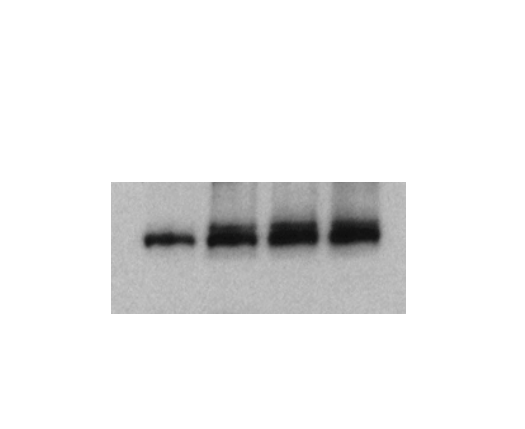
34

35

36

   1              2            3          4

SERCA2  BA



450

400

350

300

250

200

150

100

50

0

     C                                  KD1                          KD2                          KD3

\*   \*

\*

 C                                        KD1                                KD2                                KD3

Fig 6.

1 A

2

3

Input

IP: FLAG

**Human Molecular Genetics**

B

I

Input

IP:Myc

**Page 46 of 48**

4 Myc-W1

5 E-Myc

6

7 FL-SERC

8

9 E-FL

+ + - - + + - - +

- - + + - - + + -

+ - + - + - + - +

- + - + - + - + -

Myc-W1

E-Myc

FL-SERC

E-FL



+ + - - + + - -

- - + + - - + +

+ - + - + - + -

- + - + - + - +

10

11

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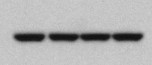
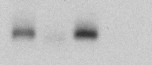
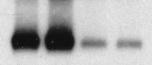
22

23 C

24

25

1 2 3 4



Input

Myc



W1

FLAG BA

1 2 3 4 5



IP

100kDa

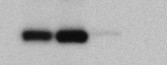
100kDa

110kDa

D

Input

1 2 3 4



I

FLAG Myc BA



IP

1 2 3 4

110 kDa

100kDa

26

27

28 SERCA2



29

30

31

32

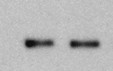
33

1 2 3 4 5 6

110 kDa

D U

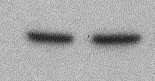
SERCA2



WFS1



BA



1 2 3 4 5

0.38 1.0



1.0 1.0

SERCA2 110 kDa

WFS1 100 kDa

**Page 47 of 48 Human Molecular Genetics**

1

2 Fig 7.

3

4

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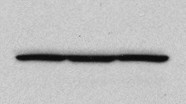
14

15

16

17

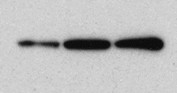
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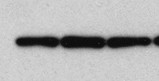
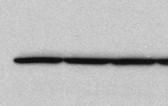
|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| 19 | 1 | 2 | 3 | 1 | 2 | 3 |
| 20 |  | | | | | |
| 21 |
| 22 |
| 23 |
| 24 |
| 25 |
| 26 |
| 27 |
| 28 |
| 29 |
| 30 |

C KD2

MG132 MG132



SERCA BA



350

300

250

SERCA expression (%)

200

150

100

50

0

\* \*

DMSO MG132\_5 MG132\_10

C KD2

Fig 8

Healthy person

**Human Molecular Genetics**

Wolfram syndrome

**Page 48 of 48**

SERCA

14

15

16

17 WFS1

18

19

20

21

22

23

SERCA

degraded

ER stress

|  |  |  |
| --- | --- | --- |
| 1 | SERCA levels controlled by WFS1 | WFS1 |
| 2 |  |
| 3 |  |
| 4 |  |
| 5 |  |
| 6 | WFS1 SERCA |
| 7 |  | Mutated WFS1 |
| 8 |  |  |
| 9 |  | ER stress |
| 10 |  |  |
| 11 |  |  |
| 12 | Physiological levels |  |
| 13 | of SERCA turnover | SERCA not |

WFS1

SERCA

compensatory Ca pumping to ER

Complex not formed degraded

SERCA

upregulation

compensatory Ca pumping to ER

24 Physiological

25 SERCA levels

26

27

28

29

30

31

32

33

34

35

36

37

38

39

40

41

42

43

44

45

46

Complex not formed

SERCA not degraded

SERCA

upregulation

In time in WFS1 depleted cells unresolvable ER stress will develop due to impairment of multiple WFS1 function.

Cell death