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Sodium-potassium ATPase beta 1 subunit is a molecular partner of Wolframin, an endoplasmic reticulum (ER) protein involved in ER stress

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Abstract

Wolfram syndrome, an autosomal recessive disorder characterised by diabetes mellitus and optic atrophy, is caused by mutations in the WFS1 gene encoding an endoplasmic reticulum (ER) membrane protein, Wolframin. Although its precise functions are unknown, Wolframin deficiency increases ER stress, impairs cell cycle progression and affects calcium homeostasis. To gain further insight into its function and identify molecular partners, we used the WFS1-carboxy-terminal domain as bait in a yeast two-hybrid screen with a human brain cDNA library. Na^+/K^+ ATPase β1 subunit was identified as an interacting clone. We mapped the interaction to the WFS1 carboxy-terminal and transmembrane domains, but not the amino-terminal domain. Our mapping data suggest that the interaction most likely occurs in the ER. We confirmed the interaction by co-immunoprecipitation in mammalian cells, and with endogenous proteins in JEG3 placental cells and neuroblastoma SKNAS cells. The expression of Na^+/K^+ ATPase β1 subunit was reduced in human WFS1 mutant fibroblasts compared with wild type, and in WFS1 silenced MIN6 pancreatic β-cells compared with wild type. Induction of ER stress in wild type cells only partly accounted for the reduced Na^+/K^+ ATPase β1 subunit expression observed. Na^+/K^+ ATPase β1 subunit is the first identified protein partner of Wolframin; the interaction is most likely to occur in the ER, and may be important for Na^+/K^+ ATPase β1 subunit expression, folding or maturation.

Introduction

Diabetes mellitus is a heterogeneous disorder characterized by glucose intolerance, and affects over 170 million people worldwide (1). It arises from a combination of absolute (type 1) or relative (type 2) insulin deficiency with a variable tissue insulin resistance (2). Recently endoplasmic reticulum (ER) stress has been identified as a novel contributor to insulin

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resistance in type2 diabetes (3) and may be involved in the loss of pancreatic β-cells that occurs in this disease (4). Wolfram syndrome (5) is characterized by childhood-onset diabetes mellitus and neurodegeneration manifesting as progressive optic atrophy, diabetes insipidus, sensorineural deafness, neuropathic bladder and cerebellar ataxia (6). The syndrome is caused by loss of function mutations in the WFS1 gene (7,8), encoding Wolframin, an ER membrane protein (9). Mutations are distributed throughout the entire gene, but with one loss of function mutation at the carboxy-terminal end (c. 2648-2651delTCTT; F883fsX950) recurring in white European populations (7, 10). Mice with a disrupted WFS1 gene show glucose intolerance and progressive pancreatic β-cell loss (11,12). This appears to be via activation of ER stress pathways, impaired cell cycle progression, and apoptosis (12,13,14).

Pancreatic β-cells have a highly developed ER, reflecting their major function of secreting insulin (15). The ER has many roles, which include post-translational modification, folding, and assembly of newly synthesized proteins such as insulin. Conditions that disturb ER function are collectively known as ‘ER Stress’, and in the β-cell may include an increased demand for insulin synthesis subsequent to insulin resistance in obesity or type 2 diabetes. At least in other cell types, the ER stress response (16,17) involves upregulation of genes encoding ER chaperone proteins which increase protein folding activity and prevent protein aggregation; translational attenuation to reduce the synthesis of new protein and prevent the further accumulation of unfolded proteins; degradation of misfolded proteins; and finally apoptosis, which occurs when severe and prolonged ER stress extensively impairs ER functions. Pancreatic β-cells are some of the most susceptible cells to ER stress, and ER stress-mediated apoptosis causes diabetes in Wolfram syndrome (14).

All 3 ER stress pathways (PERK, IRE1, ATF6) are activated by WFS1 deficiency in pancreatic β-cells (13). In addition, WFS1 expression increases in response to ER stress (18). ER stress pathways are activated by many stimuli which include inhibition of protein glycosylation, reduction of formation of disulphide bonds, calcium depletion from the ER lumen, impairment of protein transport from the ER to the Golgi, and expression of malfolded proteins (15). There is also some evidence that Wolframin functions as an ion channel or regulator of existing channels (19) and is involved in intracellular calcium homeostasis by modulating the filling state of the ER Calcium store (20). It is possible that in WFS1 deficient cells, impaired ER calcium homeostasis leads to ER stress and pancreatic β-cell apoptosis. It was also suggested that Wolframin may function in protein assembly, protein folding (e.g. proinsulin folding and processing) and/or transport out of the ER (13).

To further investigate the function of Wolframin and the mechanism of pancreatic β-cell failure in Wolfram syndrome, we undertook a yeast two-hybrid screen using the WFS1 C-terminal domain as bait in a human cDNA library. Using this and complementary approaches we identify the Na^+/K^+ATPase β-1 subunit as a novel interacting partner and show that the expression of Wolframin parallels that of Na^+/K^+ATPase β-1 subunit in a variety of settings. Decreases in Na^+/K^+ATPase activity may thus contribute to changes in β-cell mass and function in Wolfram syndrome

Results

Identification of Na^+/K^+ ATPase β1 subunit as an interacting partner for WFS1

We used the yeast two-hybrid system to screen a pre-transformed human brain cDNA library (Clontech) with the carboxy-terminal (C-terminal) domain of the human WFS1 gene (aa 652-890) as bait. The C-terminal domain is located in the ER lumen and many Wolfram patients have mutations that result in its truncation or frameshift and an extended polypeptide tail (10). We chose a human brain library as neurodegeneration is a major component of Wolfram syndrome. Approximately 6 x 10^6 independent transformants were screened, of which 8 clones were positive for α-galactosidase expression. One of the interacting clones was identified as the C- terminal domain of the Na^+/K^+ATPase β1subunit containing the coding region for amino acids 113-303 (of 303). We chose this clone for further study.

Confirmation of WFS1- Na^+/K^+ ATPase β1 interaction in mammalian cells

We confirmed the above interaction in mammalian Cos7 cells transiently co-transfected with full length WFS1 (pCMV -Myc- WFS1) and either: the C-terminal domain of β1 subunit, corresponding to the clone identified in the library (pCMV-HA-β1-C); or with full length Na^+/K^+ATPase β1subunit (pCMV-HA-β1) (Fig 1C). Using rabbit polyclonal c-myc antibody (Sigma) for immunoprecipitation, and mouse monoclonal HA antibody for immunoblotting, we detected either: a ~25kDa protein, which corresponded to the C-terminal domain of Na^+/K^+ATPase β1subunit (Fig 1A lanes 9, 10); or a 35-50 kDa protein corresponding to full length Na^+/K^+ATPase β1subunit (Fig 1A lanes 4, 5). The 35-50 kDa protein appeared to be migrating as multiple bands possibly due to different levels of glycosylation (21). The identity of these proteins was confirmed by reprobing the membrane with specific anti-Na^+/K^+ATPase β1subunit antibody (mouse, monoclonal, Sigma). In the reverse experiment, using mouse, monoclonal HA antibody (Sigma) for immunoprecipitation and polyclonal, rabbit c-myc antibody (Sigma) for immunoblotting, we detected a ~100KDa protein corresponding to Wolframin (Fig 1B lanes 4-7). Its identity was confirmed by reprobing with specific anti-Wolframin antibody (rabbit, polyclonal (22). No co-immunoprecipitation was observed in control extracts co-transfected with either empty pCMV-Myc and pCMV-HA-β1 (Fig 1A lanes 6,7) or empty pCMV-HA and pCMV-Myc-WFS1 (Fig1A lanes 2, 3, 11, 12; Fig1B lanes 2, 3). No co-immunoprecipitation of Wolframin with unrelated proteins FLAG-RasF1 or GFP was observed in this mammalian system. We concluded that the interaction between Wolframin and Na^+/K^+ATPase β1subunit is a specific interaction to Wolframin.

The COOH-terminal and transmembrane domains of Wolframin interact with Na^+/K^+ATPase β1subunit

Next we mapped the Wolframin domains interacting with Na^+/K^+ ATPase β1 subunit. We prepared a series of WFS1 deletion constructs in plasmid pCMV-Myc, tagged with c-Myc at the N-terminus, and tested their expression in Cos7 cells (Fig 2A). Wolframin (aa 1-652) lacking its C-terminus (ΔC) and C-terminal domain of Wolframin (C-; aa 652-890) were both well expressed and migrated in SDS PAGE as single bands of the expected size. Wolframin lacking its N-terminus (aa 322-890; ΔN) was expressed very weakly probably

due to instability. The N-terminal domain of Wolframin (N-; aa 1-321) was always seen with a small degradation product (Fig2A). We also attempted to prepare a Wolframin transmembrane domain (TM; aa 322-652), but never expressed a product of expected size from the plasmid. We transiently co-transfected truncated WFS1 plasmids with full length Na^+/K^+ATPase β1 subunit to Cos7 cells and co-immunoprecipitated with anti c-Myc antibody (Fig2 B-E). In each experiment we included a positive control (WFS1 wt co-transfected with β1 subunit: W x β1) and a negative control (empty vector pCMV-Myc co-transfected with β1 subunit:V x β1). The C-terminal domain (Fig 2B), but not the N-terminal domain (Fig 2C) of Wolframin co-immunoprecipitated with Na^+/K^+ATPase β1subunit as expected from our yeast two hybrid experiment. Truncated Wolframin missing its N- terminus (ΔN) was able to co-immunoprecipitate with β1 subunit, as expected (ΔN includes the Wolframin C-terminal domain). This protein was poorly expressed (again, possibly due to inherent instability, Figs 2A and 2D). Interestingly the truncated Wolframin missing its C-terminus (ΔC) was also able to interact with Na^+/K^+ATPase β1subunit (Fig 2E), which indicates that there may be an additional β1 binding domain located in the transmembrane region. In summary we demonstrated two Wolframin domains interacting with Na^+/K^+ATPase β1 subunit: the C-terminus (aa 652-890); and transmembrane domain (aa 322-652); and found that N-terminal domain is not able to interact.

Demonstration of interaction between endogenous proteins

We further confirmed the interaction between Wolframin and Na^+/K^+ATPase β1subunit by co-immunoprecipitation of endogenous proteins. Antibody to anti-Na^+/K^+ATPase β1subunit (mouse, monoclonal, Sigma) was used for immunoprecipitation, and anti-Wolframin affinity purified antibody (in house, polyclonal, sheep) was used for immunoblotting. A 100 kDa protein was detected in JEG-3 placental cells (Fig 3A), and neuroblastoma cell lines: SKNAS (Fig.3B), (both expressing high levels of Wolframin). No immunoprecipitated protein was detected in negative controls where unrelated, monoclonal anti HA antibody (Sigma) was used for immunoprecipitation (Fig 3A, B,).

Expression levels of Na^+/K^+ATPase β1subunit are reduced with absent or reduced WFS1 expression

To address the question of the possible role of Wolframin/ Na^+/K^+ATPase β1subunit interaction we first tested whether there are differences in overall expression level of Na^+/K^+ATPase β1subunit in cells expressing different levels of Wolframin. We compared the levels of Na^+/K^+ATPase β1subunit expression in wild type (wt) fibroblasts with fibroblasts from a Wolfram patient carrying a homozygous W700X mutation (23). This mutation leads to truncation of the major portion of the hydrophilic C-terminus and results in almost undetectable levels of Wolframin in fibroblasts from this patient, caused probably by instability of this protein. We found that the expression level of Na^+/K^+ ATPase β1 subunit in this Wolfram patient was significantly lower than in wild type fibroblasts: on average the decrease was 91% ±2, and varied from 88-93% (n=5; Fig4A). A similar decrease in Na^+/K^+ ATPase β1 expression was observed in fibroblasts from another patient carrying a homogenous C insertion (1029insC/1029insC) which results in a frameshift and introduces a premature stop at codon 395 (data not shown). This mutation also results in undetectable levels of Wolframin in fibroblasts (22).

To determine if this difference in Na^+/K^+ATPase β1subunit expression was a consequence of altered Wolframin expression, we studied this in another system: stable MIN6 cell lines with reduced Wolframin expression (a kind gift from Professor Alan Permutt). The MIN6 knock downs were constructed using RNA interference in mouse MIN6 insulinoma cells as described previously, and either 50% (KD1) or 70% (KD2) reduction in Wolframin expression was demonstrated (12). We observed a significant reduction in Na^+/K^+ ATPase β1 subunit expression in both KD1 and KD2 cell lines compared to the control: KD2 (70% knock down) mean (SD) 64% ± 10 decrease in Na^+/K^+ ATPase β1 subunit (range 49-79%, n=5; Fig4B); KD1 (50% knock down) mean (SD) 43%±6, (range 20-65%, n=5). We considered two possible explanations for our observations: first, that it may be due to an indirect effect of induction of ER stress in WFS1 mutant cells, and second, that Wolframin may be necessary for proper Na^+/K^+ ATPase β1 subunit folding or maturation.

To explore these possibilities we induced ER stress in wild type fibroblasts and wild type MIN6pSuper (12) cells with thapsigargin. We then compared the expression of Na^+/K^+ ATPase β1 subunit in untreated cells (T_0) and cells treated with thapsigargin for 24h (T_{24}). The ER stress response was measured by detecting the ER stress marker CHOP (Ddit3 for mice), barely detectable under physiological conditions but strongly induced in response to ER stress (15). The increased level of CHOP after 24 hours of treatment with thapsigargin demonstrated that the ER stress response was induced in both wild type MIN6 cells and wild type fibroblasts (Fig 5A,B). In wild type fibroblasts, after 24 hours treatment with thapsigargin we observed a decrease in Na^+/K^+ ATPase β1 subunit expression in comparison to T_0 (mean decrease 33% ± 9, n=7; range 20-39%; Fig 5A). However this decrease was smaller than that observed in WFS1 mutant fibroblasts (90%). In thapsigargin treated MIN6 pSuper, the levels of Na^+/K^+ ATPase β1 subunit expression were also decreased (mean decrease 50%±8, n=5; range 37-59%; Fig 5B). This decrease was comparable to that observed in MIN6 knock down cells (43% decrease in KD1, 64% decrease in KD2). These results suggest that the decreased expression of Na^+/K^+ ATPase β1 subunit in MIN6 knock down cells may be secondary to induction of ER stress. However, in fibroblasts, the expression of Na^+/K^+ ATPase β1 subunit is also reduced in WFS1 mutants compared to thapsigargin treated wild type cells (90% vs 33%), suggesting that induction of ER stress response alone is unlikely to be responsible for this change.

Discussion

We used the carboxy-terminal domain of WFS1 to screen for interacting partners in a human brain cDNA library, and identified Na^+/K^+ ATPase β1 subunit, confirming the interaction in transfected Cos7 cells, and between endogenous proteins in placental and neuroblastoma cell lines. This is the first identified protein partner of Wolframin. Furthermore, we found reduced Na^+/K^+ ATPase β1 subunit expression in WFS1 mutant fibroblasts and WFS1 knock-down MIN6 pancreatic β-cells. Induction of ER stress in wild type cells only partly accounted for the reduced Na^+/K^+ ATPase β1 subunit expression observed. Given Wolframin's predominant localisation to this compartment, it seems reasonable to propose that the interaction with the Na^+/K^+ ATPase β1 subunit most likely occurs in the ER, and may thus be important for Na^+/K^+ ATPase β1 subunit expression, folding or maturation.

We demonstrate that the Wolframin-Na^+/K^+ ATPase β1 interaction occurs with the Wolframin C-terminal domain (aa 652-890), and the transmembrane region (aa 322-655), but not the N-terminal domain (aa 1-322) (Fig.2). The localisation and membrane topology of Wolframin is known: it is an ER membrane protein (9) with the C- terminal domain located in the ER lumen, and N-terminal domain in the cytoplasm with 9 membrane spanning domains and loops facing both sides of the ER membrane (22,24). It assembles into higher molecular weight complexes of ~400kDa in the ER membrane (22). The mature sodium pump (α and β subunits) is located in the plasma membrane; the N-terminal amino acids (1–34) of Na^+/K^+ ATPase β1 subunit are in the cytoplasm; amino acids 36-62 form the signal-anchor; and the C-terminal domain (aa 63-303) is located extracellularly. However, the sodium pump is present transiently in the ER during maturation and assembly in common with other oligomeric membrane proteins (25). The ER membrane may be juxtaposed to the plasma membrane, allowing interactions between ER and plasma membrane proteins (e.g. the interaction of Na^+/K^+ ATPase β1 subunit with inositol 1,4,5-*tris*phosphate receptor (InsP3R), (26). However our mapping data showed that the C-terminal domain of Wolframin (facing the ER lumen) is sufficient for interaction with Na^+/K^+ ATPase β1 subunit and the cytoplasmic N-terminal Wolframin domain did not interact; we therefore conclude that the ER lumen is the most likely site for this interaction (Fig6).

We then considered the hypothesis that Wolframin interacts with Na^+/K^+ ATPase β1 subunit to facilitate the correct assembly and folding of the sodium pump. Wolframin has been proposed to be involved in the folding and processing of another protein, proinsulin, in the ER of pancreatic β-cells (13); electron microscopy of pancreatic β-cells from mice with a conditional deletion in WFS1 showed abnormal, dilated ER with decreased insulin secretory granules (12). In addition, abnormal processing of vasopressin precursors has been reported in Wolfram syndrome (27). In support of a role in protein folding and maturation, the WFS1 promoter contains a conserved sequence highly similar to the known ER Stress Response Elements (ERSE) found in ER chaperone proteins such as Bip/GRP78 and GRP94 (28,29,30). These proteins, like Wolframin, are upregulated during ER stress; ER chaperone proteins increase protein folding activity and prevent protein aggregation (15). The Na^+/K^+ ATPase β1 subunit has been shown to be essential for the maturation and assembly of the sodium pump in the ER, and is transported to the plasma membrane already assembled with the catalytic α subunit (21). In the ER, the β-subunit is necessary for the stability of the newly synthesised α-subunit: it may shield a degradation signal in the M7/M8 loop of the α-subunit and thus protect the α-subunit from ER associated degradation (ERAD) (31). It is possible that Wolframin facilitates the assembly of the α- and β-subunits into fully formed Na^+/K^+ ATPase in preparation for translocation out of the ER. This may be part of a wider role of Wolframin to facilitate the correct folding of oligomeric proteins.

We examined this hypothesis against our observations of reduced expression of Na^+/K^+ ATPase β1 subunit in human WFS1 mutant fibroblasts (90% reduction) compared with wild type, and in WFS1 silenced MIN6 pancreatic β-cells compared with wild type (60% reduction) (Fig 4). The reduction in Na^+/K^+ ATPase in WFS silenced cells is unlikely to be explained by off-target effects of the WFS siRNA, as a blast search revealed only WFS1 sequence. If Wolframin has a role in protein folding and maturation, then in the absence of Wolframin, unassembled Na^+/K^+ ATPase β1 subunits may accumulate in the ER, leading to

degradation by ERAD, and reduced overall expression of Na^+/K^+ ATPase. An alternative explanation is that the reduced expression is secondary to attenuation of protein translation as part of the ER stress response induced by the absence of Wolframin (13). We therefore induced ER stress in wild type (w/t) fibroblasts and wild type MIN6 cells. We found that in w/t MIN6 pSuper, after 24 hours treatment with thapsigargin the levels of Na^+/K^+ ATPase β1 subunit expression were reduced by 50% in comparison to T_0 (Fig. 5B;). This was comparable to the reduced expression observed in KD2 WFS1 knock down cells (64%, Fig. 4B). However, in thapsigargin treated fibroblasts the levels of Na^+/K^+ ATPase β1 subunit expression were reduced by 30%, compared with a 90% reduction of Na^+/K^+ ATPase β1 subunit in W700X null mutant fibroblasts (Fig.4A, 5A). This result suggests that in MIN6 knock down cells, the reduced expression of Na^+/K^+ ATPase β1 subunit may in part be secondary to induction of ER stress. However, in WFS1 mutant fibroblasts, the greater reduction in Na^+/K^+ ATPase β1 subunit expression is only partly explained by induction of ER stress, and may also be a direct effect of the absence of Wolframin. The smaller reduction in Na^+/K^+ ATPase β1 subunit expression in MIN6 knock down cells may reflect the incomplete suppression of Wolframin expression. The observations in WFS1 mutant and w/t fibroblasts are consistent with a proposed function for WFS1 in protein folding and maturation/membrane insertion.

We then considered an alternative hypothesis: that Na^+/K^+ ATPase β1 subunit acts as a chaperone for Wolframin. The Na^+/K^+ ATPase β1 subunit chaperones Na^+/K^+ ATPase α subunits, and has also been shown to transiently associate with the sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA) in *Xenopus laevis* oocytes (32). The SR Ca^{2+} ATPase and the Na^+/K^+ ATPase α subunits have similar membrane topologies, and are both assisted by association with the β-subunit for correct packing into membranes. Because the M7/M8 loop of the SR Ca^{2+} ATPase does not contain the conserved 4-amino acid sequence of the α subunit of Na^+/K^+ ATPase, the association is not strong enough to associate permanently. Wolframin is thought to modulate the activity of the SERCA Ca^{2+} pump: ER Ca^{2+} content was decreased in WFS1-knockdown cells; increased in WFS1-overexpressing cells; and Ca^{2+} flux in both cells lines inhibited by the SERCA inhibitor cyclopiazonic acid (20). It is possible that the interactions between Na^+/K^+ ATPase β1 subunit, and both Wolframin and SR Ca^{2+} ATPase, are transiently required for their maturation and membrane insertion. In conditions of ER stress, Wolframin may facilitate the function of SR Ca^{2+} ATPase to sequester Ca^{2+} into the ER calcium pool. In the absence of Wolframin, SR Ca^{2+} ATPase function may be decreased, allowing ER calcium levels to be depleted and triggering ER stress. This could trigger a reduction in Na^+/K^+ ATPase β1 subunit expression, although such a scenario does not explain the greater reduction we observed in WFS1 mutant fibroblasts. Further time course studies on interactions between these three proteins may reveal if Na^+/K^+ ATPase β1 subunit links the maturation and/or functions of Wolframin and SR Ca^{2+} ATPase.

Finally we considered the consequences of our findings of reduced Na^+/K^+ ATPase β1 subunit expression in WFS1 mutant and knock down cells, and with induced ER stress in wild type cells. These findings imply significantly reduced expression of Na^+/K^+ ATPase on the plasma membrane in these cell lines, which would be expected to affect insulin secretion. The effect of the Na^+/K^+ ATPase inhibitor Ouabain on pancreatic islets has been

studied (33). Ouabain was found to paradoxically inhibit the elevation of intracellular ATP concentration in response to glucose, and thus to reduce insulin secretion. Correspondingly, glucose-stimulated insulin secretion and changes in intracellular free Ca^{2+} concentration were defective in islets from WFS1 knockout mice (11), and ER Ca^{2+} concentrations were decreased in cell lines silenced for WFS1 (20). On the other hand, Riggs and colleagues (12) reported that insulin secretion was decreased from the MIN6 WFS KD cells used in the present study only proportionately with the decrease in cellular insulin content. These observations confirm that the role of WFS1 in the acute triggering of insulin release requires further study.

The activity of Na^+/K^+ ATPase is known to be reduced in many tissues of streptozotocin-induced diabetic animals, and in the red blood cell membranes of type 1 diabetic (insulin deficient) humans (34). This has been proposed to be a secondary effect of reduced C-peptide levels (where C-peptide concentration reflects that of insulin). Na^+/K^+ ATPase deficiency has been identified as a contributor to apoptosis and neural degenerative disease, of which Wolfram syndrome is one example. To the best of our knowledge, reduction in Na^+/K^+ ATPase expression has not previously been reported in ER stress. Our findings suggest that Na^+/K^+ ATPase insufficiency may contribute to the known mechanisms of β-cell apoptosis, and possibly regulated insulin secretion, reported in Wolfram syndrome.

Materials and Methods

Yeast two-hybrid system

Yeast two-hybrid screening was performed using MATCHMAKER system 3 (BD-Clontech) according to manufacturer instructions. The C-terminal domain of WFS1 (aa 652-890) was used as a bait to screen a pretransformed brain cDNA library (Clontech). Positive clones were retransformed to yeast to confirm a positive interaction and were sequenced using automated DNA sequence analysis (ABI) and homologies identified using National Center for Biotechnology Information BLASTN/BLASTX.

Plasmids

Yeast two-hybrid bait: the C-terminal WFS1 cDNA (aa 652 to 890) was amplified on the IMAGE 412765 with primers: 9 and 10 (see below) and cloned into pGBKT7 (Clontech).

pCMV-Myc-WFS1: full length WFS1 cDNA (aa 1-890) was amplified on IMAGE 412765 with primers 1 and 2 and cloned in pCMV-Myc (Clontech). Full length Na/K ATPase β1 subunit: the cDNA was amplified on IMAGE 3506311with primers 7 and 8 and cloned in pCMV-HA (Clontech). The C-terminal part of Na^+/K^+ATPase β1subunit was subcloned as an EcoRI-XhoI fragment to pCMV-HA from the yeast two hybrid library prey clone (in pACT2). Truncated WFS1 clones were amplified on pCMV-Myc-WFS1 as a template as follows: N-: with primers 1 and 6; C- with primers 5 and 2; TM with primers 3 and 4; ΔC with primers 1 and 4; and ΔN with primers 3 and 2 and cloned in pCMV-Myc.

Primers

1. 5’ GCAGG AATTC GGATG GACTC CAACA CTGCT CC

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2. 5’ GCAGG GTACC TCAGG CCGCC GACAG GAATG

3. 5’ GAGAA TTCGG CACCA CATCA ACGCG CTCAT

4. 5’ GCAGG GTACC CACAT AGAAC CAGCA GAACA

5. 5’ GAGAA TTCGG TACCG CTCAG AGGGC ATGAA

6. 5’ GACGG GTACC CGTGG GGATG ATGGT GGACA

7. 5’GGGAATTCCCATGGCCCGCGGGAAAGCCAA

8. 5’ GGCTCGAGTGTGATCAGCTCTTAACTTC

9. 5’ GCAGG AATTC CCGGG GTACC GCTCA GAGGG CATG

10. 5’ GCAGG GATCC TCAGG CCGCC GACAG GAATG GGAAG

Cell lines

Cos7 cells, JEG3 placental cell line, neuroblastoma SKNAS cells, and human fibroblasts were grown in DMEM (4500mg/L L-glucose, L-glutamine and pyruvate, Invitrogen) with 10% FCS, penicillin (25000 UI), streptomycin (25 000μg^{-1}), glutamine and non essential amino acids. MIN6 cells (MINpSuper, WFS1KD50,WFS1KD70 (12) were grown in DMEM (4500mg/L L-glucose, L-glutamine and pyruvate) with 15% FCS, penicillin (25000 UI), streptomycin (25 000μg^{-1}), glutamine, non essential amino acids, 5µl/1L of medium β-mercaptoethanol and geneticin 200µg/ml. Transient transfections were performed using FUGENE transfection reagent (Roche) according to the manufacturers’ instructions. ER stress was induced with thapsigargin. Cells were serum starved for 14 hours before the experiments. Thapsigargin (1.5µmole/l) in DMSO was added to the medium. Control T_0 was harvested immediately; thapsigargin treated cells were harvested after 24 hours in Laemelli buffer and resolved on SDS PAGE gel.

Anti WFS1 Antibodies

A peptide corresponding to the N-terminal WFS1 sequence: SLEQERSERPRAPGPQAGPGPGVRDJ was used to generate polyclonal antibodies in sheep (The Binding Site, University of Birmingham). The antibody was subsequently affinity purified on controlled pore glass affinity columns (Alta Biosciences).

Immunoprecipitation

Transiently transfected Cos7 cells were harvested by scraping in RIPA buffer (50mM Tris pH8, 150mM NaCl, 0.1% SDS, 1mM EDTA, 0.5% deoxycholate, 1% Igepal), sonicated 2x10 sec, spun down at 4ºC and supernatant collected. For co-immunoprecipitation ~200µg of each extract was precleaned with protein A agarose beads (Sigma).

20 µL of c-Myc rabbit polyclonal antibodies (Sigma) or HA mouse monoclonal antibodies (Sigma) was added to cleaned lysate and incubated overnight rotating at 4ºC. The same amounts of either rabbit serum fraction normal (Dako) or monoclonal GFP antibody (CRUK

UK) were used as negative controls. 20μl of precleaned Protein A agarose beads were added next morning and extracts were incubated for 2 hours rotating at 4ºC. The complexes were precipitated by spinning for 2 min at 13000 rpm, and washed 3-4 x with RIPA buffer. After a final spin, 30μl of 2x Laemelli loading buffer was added and samples were run on 10% SDS PAGE gel. For precipitation of endogenous proteins ~ 400μg of either JEG3 or SKNAS cell extracts was used and precipitated with 40μl of Na/K ATPase β1 mouse monoclonal antibody (Sigma) or mouse monoclonal HA (Sigma) as a negative control.

Immunoblotting

Samples were resolved on 10% SDS PAGE, at 120V with Rainbow marker (GE Healthcare Life Sciences) run alongside to estimate the size. Samples were transferred to Hybond P membrane (Amersham Biosciences) by electroblotting at 80V for 1 hour in standard transfer buffer. Antibodies were used overnight at 4ºC at the following concentrations: anti C-myc mouse monoclonal (Sigma) 1:1000, C-myc rabbit polyclonal (Sigma) 1:1000, HA mouse monoclonal (Sigma) 1:1000, HA mouse monoclonal (Roche) 1:500, Na/KATPase β1 mouse monoclonal (Sigma) 1:5000, rabbit, polyclonal Na/K ATPase β1 subunit (Upstate) 1:5000, anti WFS1 rabbit polyclonal (22) 1:5000; anti N-WFS1 (sheep, polyclonal, affinity purified, The Binding Site, university of Birmingham) 1:5000, mouse, monoclonal anti CHOP (Affinity Bioreagents) 1:500, mouse, monoclonal anti β actin (Sigma) 1:15000 in 5%milk in PBS/Tween. Secondary antibodies: anti rabbit, sheep or mouse (Dako) were used at 1:20 000 in 5% milk in PBS/Tween, incubated for 1hr at RT. Immunoblots were developed with Amersham ECL Plus western blotting detection system (GE Healthcare). Quantitative analysis of the altered expression of Na/K ATPase β1 subunit was performed by measuring Integrated Optical Density (IOD) using the programme LabWorks.

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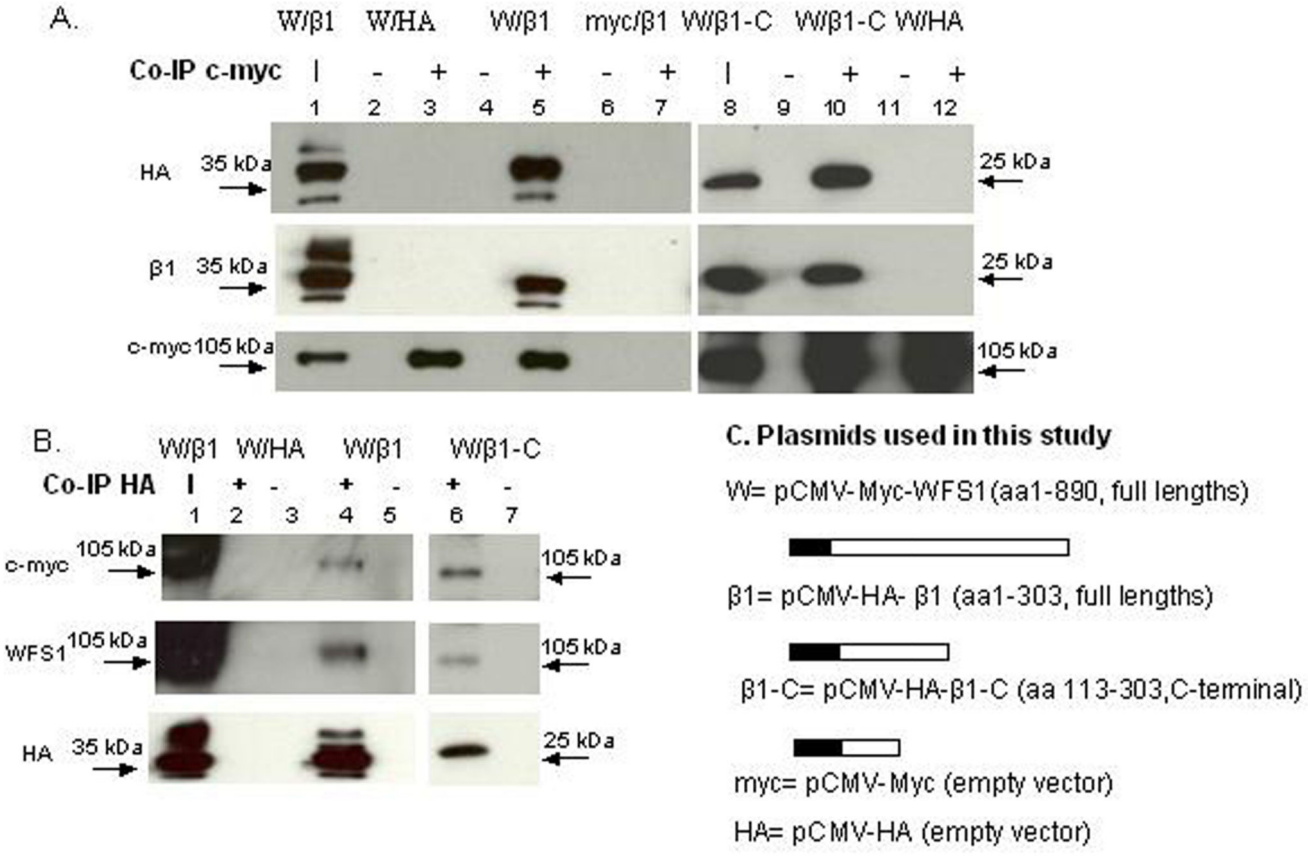

Figure 1. Co-immunoprecipitation of WFS1 and Na^+/K^+ ATPase β1 subunit cotransfected to Cos7 cells.

WFS1 interacts with both full length Na^+/K^+ ATPase β1 subunit (A lanes 4,5; B lanes 4,5) and with β1 C-terminal domain (A lanes 9, 10; B lanes 6,7)

A: Co-immunoprecipitation with C-myc antibody (polyclonal, Sigma), detection with HA antibody (monoclonal, Sigma). Below, reprobing with anti Na^+/K^+ ATPase β1 antibody (monoclonal, Sigma) to confirm identity of the clone, and with anti C-myc antibody (monoclonal, Sigma) to show the input.

B: Co-immunoprecipitation with anti-HA antibody (monoclonal, Sigma), detection with anti C-myc antibody (polyclonal, Sigma). Below, reprobing with WFS1 antibody (polyclonal, S. Hofmann) to confirm identity of the clone and with anti HA monoclonal antibody (Roche) to visualise the input.

C: Diagram showing plasmids used in this experiment, black rectangle: Myc tag.

-: negative control, immunoprecipitation with either rabbit serum fraction normal (A) or unrelated monoclonal antibody anti GFP (CRUK) (B).

+: immunoprecipitation with indicated antibody.

I: Input, 5% of lysate used for immunoprecipitation.

W= C-myc tagged full length WFS1(pCMV-Myc-WFS1), HA- empty vector pCMV- HA, myc= empty vector pCMV-Myc, β1= HA tagged, full length Na/K ATPase β1 subunit (pCMV-HA-β1), β1-C= HA tagged C-terminal domain of Na/K ATPase β1 subunit (pCMV-HA-β1-C).

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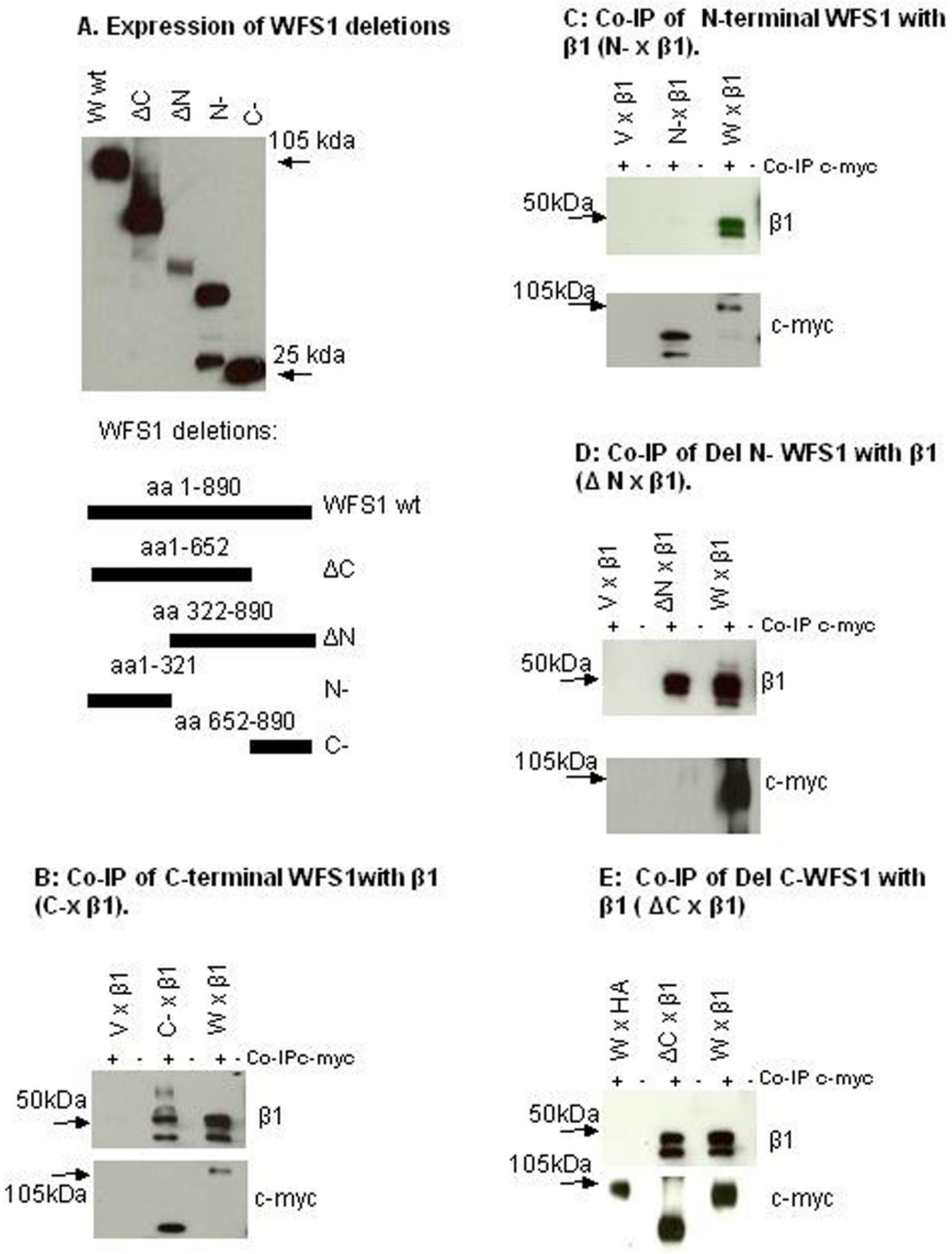

Figure 2. Mapping of WFS1 domains interacting with Na^+/K^+ ATPase β1 subunit.

A. Expression of WFS1 deletions in Cos7 cells. Each deletion construct was transfected into Cos7 cells. 20μg of protein extract was loaded in each lane. Below, diagram illustrating amino acids content of deletion constructs.

B-E: Co-immunoprecipitation of WFS1 deletions and β1 subunit with c-myc antibody. For each experiment a positive control (WFS1 full length; W x β1) and a negative control (empty vector: V x β1) are included.

B. Co-IP of C-terminal WFS1 domain with Na^+ K^+ ATPase β1 subunit (C x β1). C-terminal domain co-immunoprecipitates with β1.
C. Co-IP of N-terminal WFS1 domain with Na^+ K^+ ATPase β1 subunit (N x β1). N-terminal domain is not able to co-immunoprecipitate β1 subunit.
D. Co-IP of ΔN (WFS1 with missing N-terminal domain) with β1 subunit (ΔN x β1). ΔN is able to interact with β1 subunit although the protein is very unstable.
E. Co-IP of ΔC (WFS1 with missing its C-terminus) with β1 subunit. Interestingly this fragment of WFS1 is able to co-immunoprecipitate with β1 subunit (ΔC x B1).
+: immunoprecipitation with c-Myc antibody (polyclonal, Sigma);
-: negative control; immunoprecipitation with rabbit serum fraction normal (negative control).
Below every panel the same membrane is shown reprobed with c-Myc to illustrate the input.

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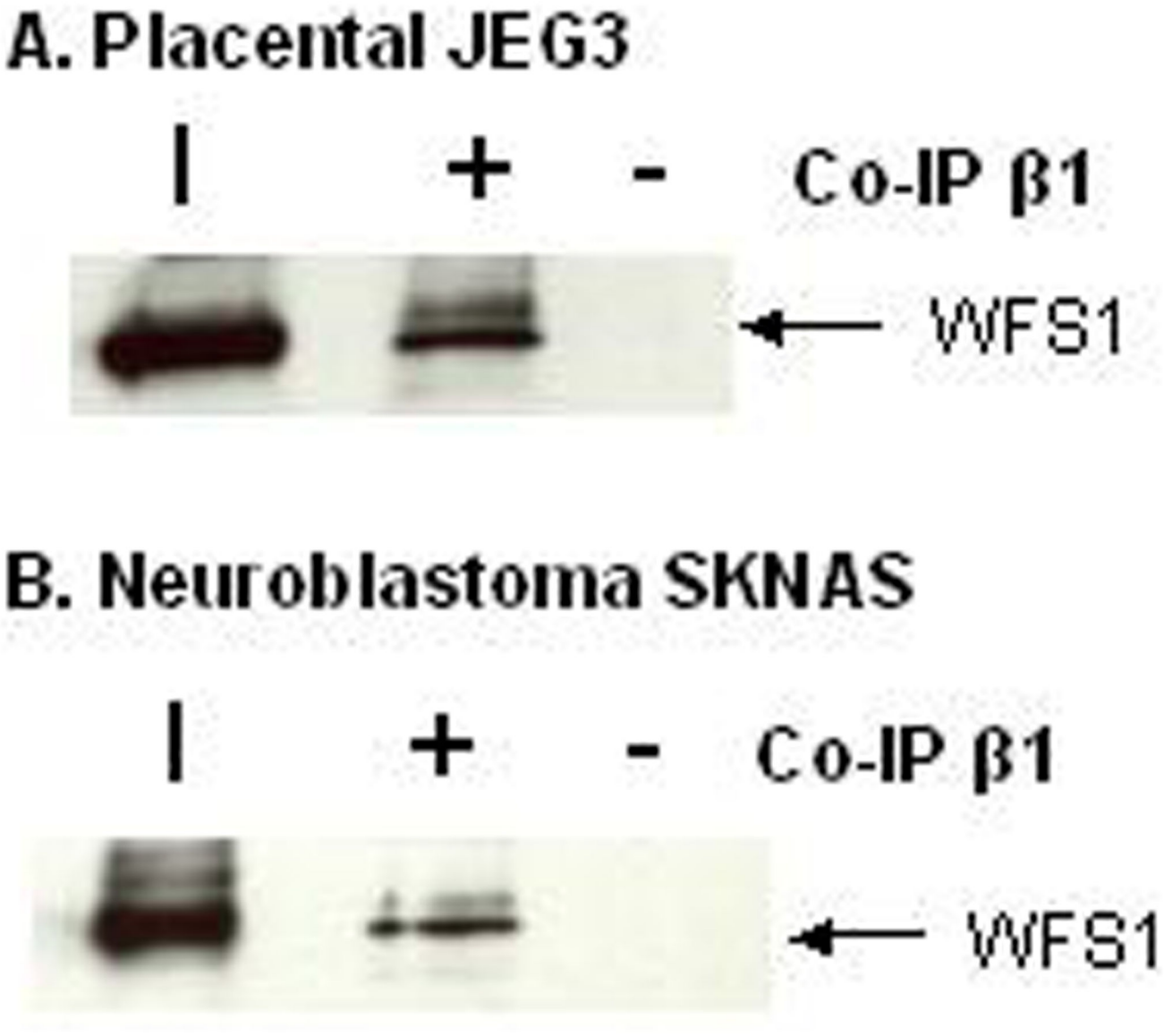

Fig 3. Co-immunoprecipitation of endogenous WFS1 and Na^+/K^+ ATPase β1 subunit.

A: Co-IP from JEG3 placental cell line expressing high level of WFS1.

B: Co-IP from SKNAS neuroblastoma cells expressing high level of WFS1

I: input,; 2%. +: co-immunoprecipitation with monoclonal anti Na^+/K^+ ATPase β1 subunit antibody (Sigma), -: negative control; co-immunoprecipitation with monoclonal anti HA antibody (Sigma). Detection with anti WFS1, sheep polyclonal antibody.

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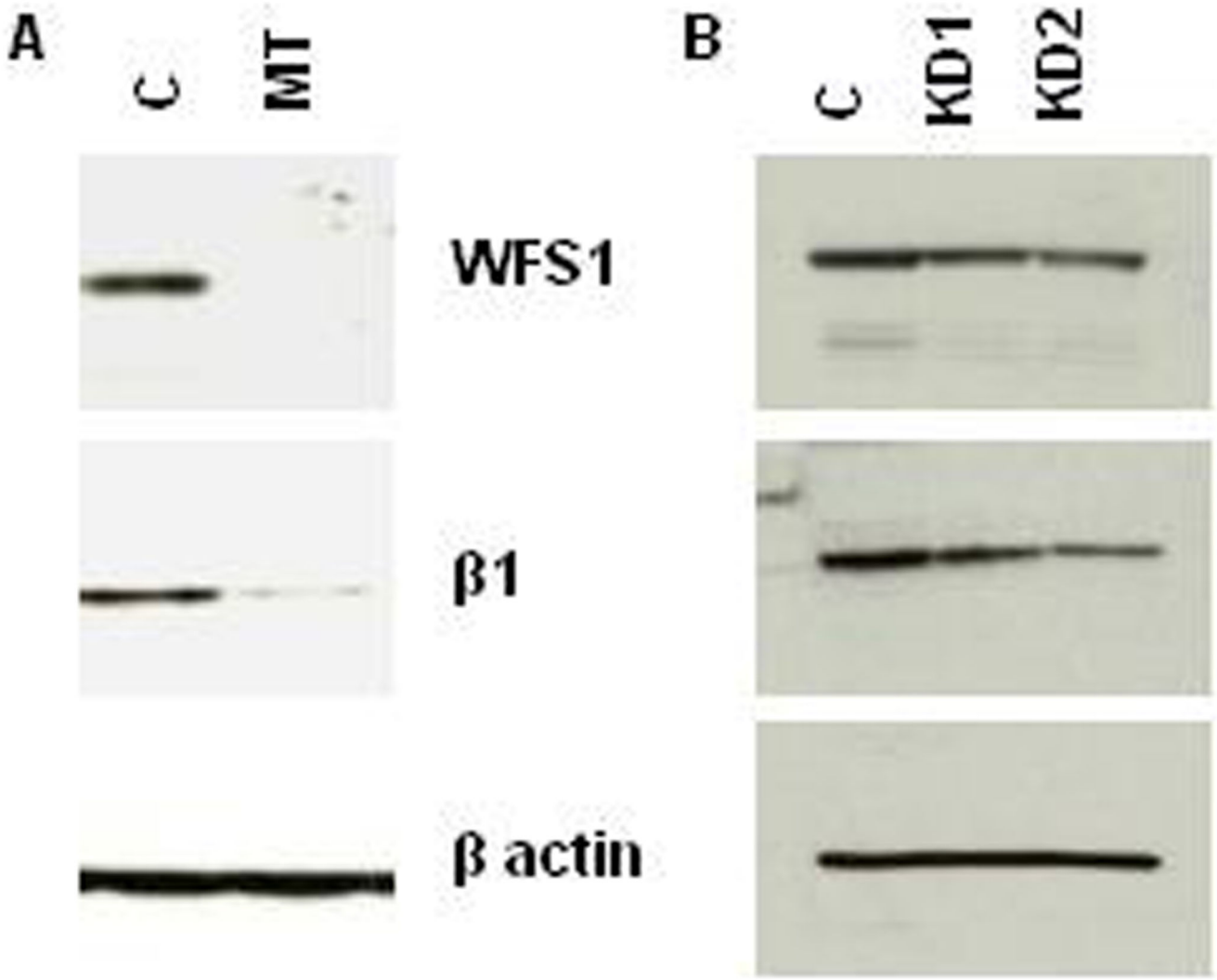

Fig4. Expression of Na^+/K^+ ATPase β1 subunit in wild type and Wolfram patient's fibroblasts and in WFS1 knock downs in MIN6 cells.

A. Expression in fibroblasts; C-control (wt WFS1 fibroblasts), MT- W700X patient carrying null mutation in WFS1. The mean decrease in β1 expression in mutant fibroblasts was 91% (SD 2, range 93-98%; n=5) (LabWorks).

B. Expression in MIN6 knock downs; C-control (MINpSuper); KD1- MIN6 with WFS1 expression silenced ~ 50%; KD2- MIN6 with WFS1 expression silenced ~70% (12). The mean decrease in β1 expression in KD2 cells was 64% (SD 10, range 49-79%; n=5). The mean decrease in β1 expression in KD1 cells was 43% (SD 6, range 20-65%; n=5).

β1- Na^+/K^+ ATPase β1 subunit.

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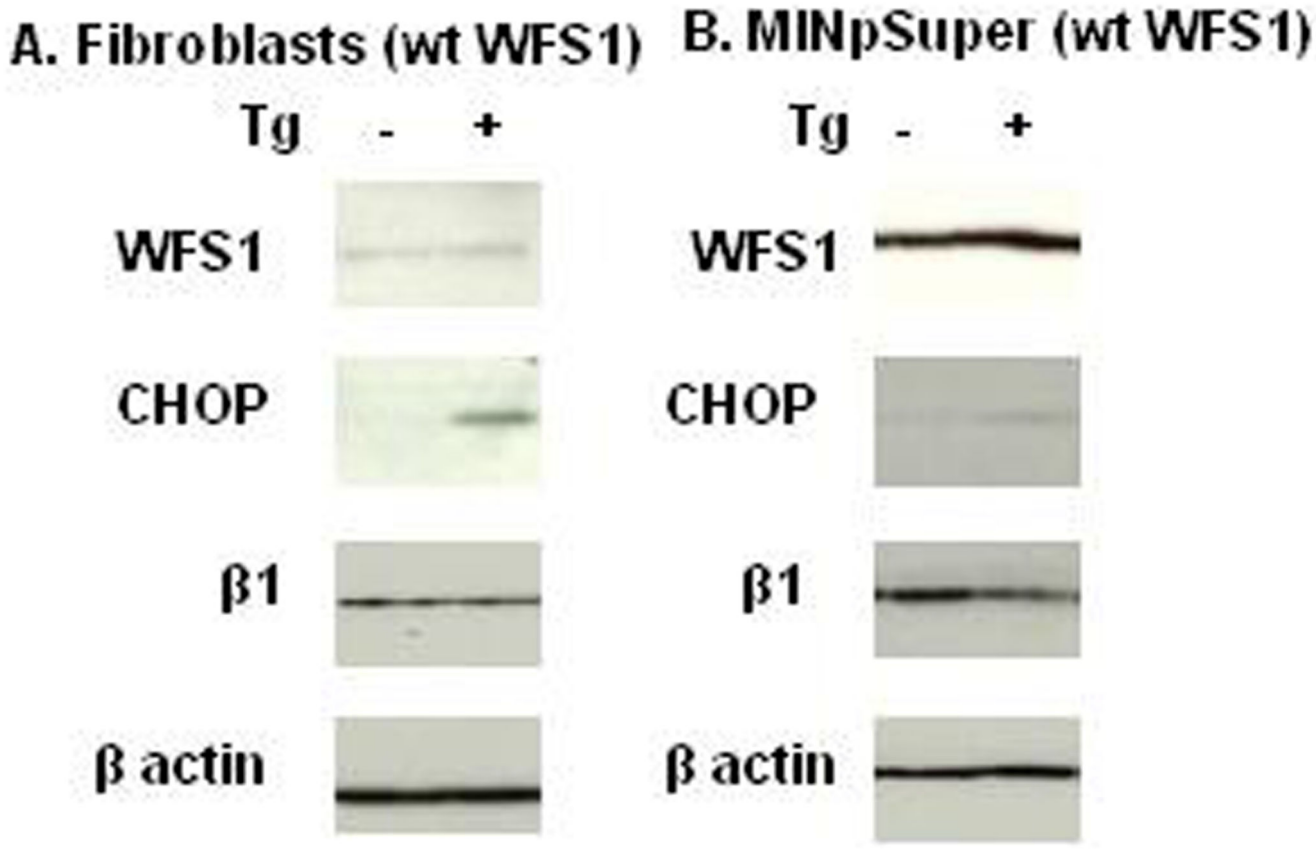

Fig5. Expression of Na^+/K^+ ATPase β1 subunit in WFS1 positive fibroblasts and MIN6 cells after thapsigargin induced ER stress response.

The ER stress was induced as shown by induction of ER stress marker CHOP.

A. Induction of ER stress response in WFS1 positive fibroblasts. The mean decrease in β1 expression was 33% (SD 9, range 20-39%; n=7).

B. Induction of ER stress response in Min6pSuper (WFS1 positive control for silencing experiments (12). The mean decrease in β1 expression in thapsigargin treated cells was 50% (SD 8, range 37-59%; n=5).

Tg- thapsigargin. Serum starved cells were treated with 1.5 μmoles/l of thapsigargin diluted in DMSO. T_0 – cells were harvested immediately after addition of thapsigargin, T_{24} – cells were harvested after 24 hours treatment with thapsigargin. The increased level of ER stress marker CHOP shows that ER stress was induced.

β1- Na^+/K^+ ATPase β1 subunit.

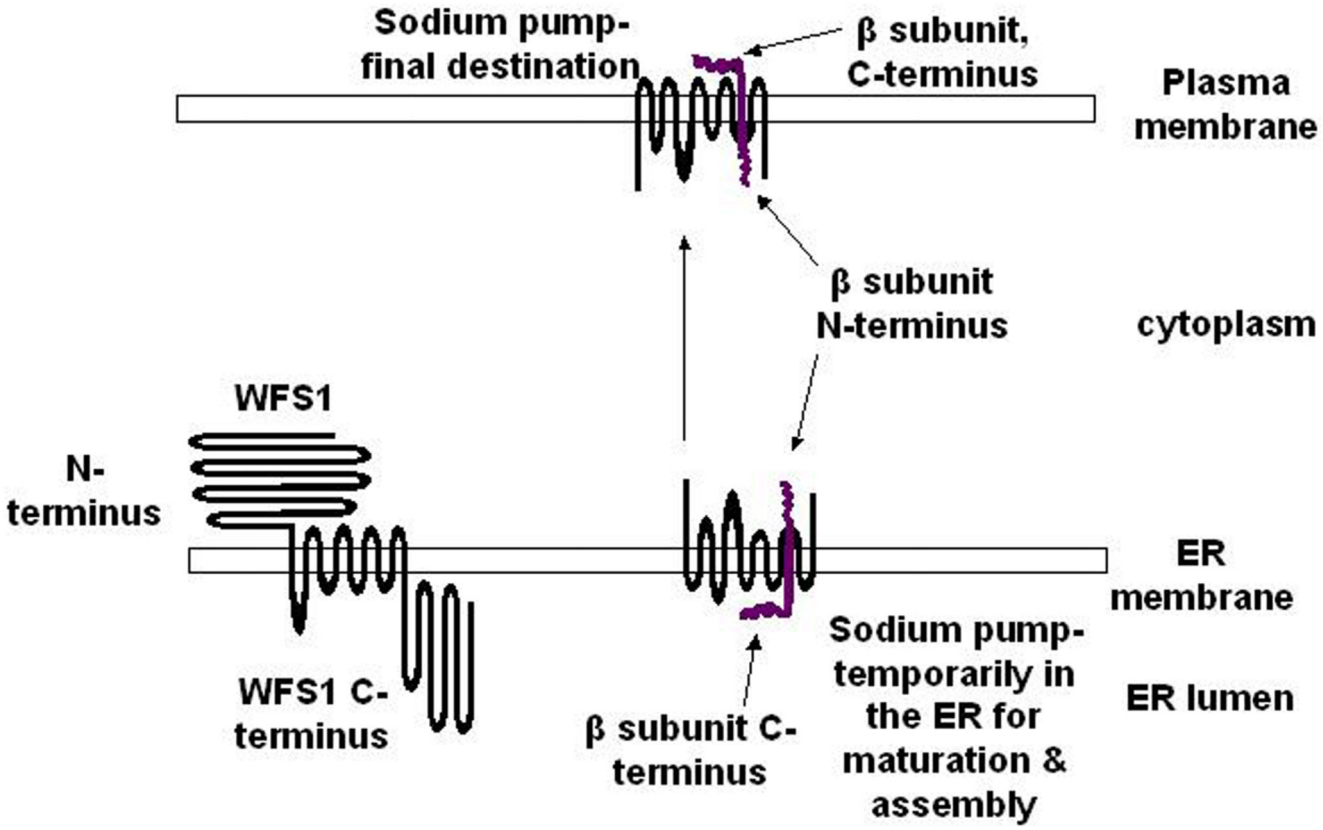

Fig6. Localisation of WFS1 and its interacting partner Na^+/K^+ ATPase β1 subunit in the cell.

The final destination for the sodium pump is the plasma membrane; however it is transiently present in the ER during its maturation and assembly of its subunits (α and β). WFS1 is an ER membrane protein with cytoplasmic N-terminus and luminal C-terminus. The most likely compartment where the WFS1-Na^+/K^+ ATPase β subunit interaction can take place is the ER (see Discussion).

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