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Constitutive ER stress and Wolfram syndrome

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A Novel Heterozygous Mutation of the *WFS1* Gene Leading to Constitutive Endoplasmic Reticulum Stress is the Cause of Wolfram Syndrome

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Abstract

Background: Wolfram syndrome (WS) is a disorder characterized by the association of insulin-dependent diabetes mellitus (DM), diabetes insipidus, deafness, and optic nerve atrophy. WS is caused by *WFS1* mutations encoding WFS1 protein expressed in endoplasmic reticulum (ER). During ER protein synthesis, misfolded and unfolded proteins accumulate, known as “ER stress”. This is attenuated by the unfolded protein response (UPR), which recovers and maintains ER functions. Because WFS1 is a UPR component, mutant WFS1 might cause unresolvable ER stress conditions and cell apoptosis, the major causes underlying WS symptoms. We encountered an 11-month old Japanese female WS patient with insulin-dependent DM, congenital cataract and severe bilateral hearing loss.

Objective: Analyze the *WFS1* and functional consequence of the patient WFS1 *in vitro*.

Results: The patient *WFS1* contained a heterozygous 4 amino acid in-frame deletion (p.N325_I328del). Her mutant WFS1 increased GRP78 and ATF6α promoter activities in the absence of thapsigargin, indicating constitutive ER stress and nuclear factor of activated T-cell reporter activity, reflecting elevated cytosolic Ca^{2+} signals. Mutant transfection into cells reduced mRNA expression levels of sarcoplasmic/endoplasmic reticulum Ca^{2+} transport ATPase 2b (SERCA2b) compared with wild type. Because SERCA2b is required for ER and cytoplasmic Ca^{2+} homeostasis, decreased SERCA2b expression might affect ER Ca^{2+} efflux, causing cell apoptosis.

Conclusion: A novel heterozygous mutation of *WFS1* induced constitutive ER stress through ATF6α activation and ER Ca^{2+} efflux, resulting in cell apoptosis. These results provide new insights into the roles of WFS1 in UPR and mechanism of monogenic DM.

Key words

Wolfram syndrome, *WFS1*, endoplasmic reticulum, ER stress, calcium

Introduction

Wolfram syndrome (WS, OMIM 222300), also known as DIDMOAD**,** is defined by the association of diabetes insipidus (DI), early-onset insulin-dependent diabetes mellitus (DM), progressive optic atrophy (OA) and sensorineural deafness (D) (1). This disease is mainly caused by mutations of *WFS1*, located on chromosome 4p16.1 (2). Since DM presents at the mean age of 6 as the first manifestations of WS (1), this syndrome is classified as the child-onset monogenic diabetes (3). The most frequent causes of morbidity and mortality are neurological disorders and urinary tract complications (4, 5).

WFS1 encodes a 100-kDa protein called WFS1 (also called wolframin). WFS1 is a hydrophobic and tetrameric protein with nine transmembrane segments and large hydrophilic regions at both termini (2, 4). This protein is expressed in various tissues such as the pancreas, brain, bone, muscles, lungs, liver, kidneys, and is a resident component of the endoplasmic reticulum (ER) membrane (2). Pancreatic islet β-cells are the major site of WFS1 expression.

During protein synthesis in the ER, an accumulation of misfolded and unfolded proteins occurs at a certain frequency (known as “ER stress”). ER stress is attenuated by activation of the unfolded protein response (UPR), which recovers and maintains the ER functions. Because WFS1 is a known component of the UPR, mutant WFS1 results in unresolvable ER stress conditions and cell apoptosis (6, 7), which is considered to be the major mechanism underlying the development of the symptoms in WS.

To date, more than 170 mutations of *WFS1* have been reported, the majority of which are distributed throughout the coding sequence and no hot spots have been identified (4). *WFS1* mutations are generally identified on both alleles, and thus WS is thought to be an autosomal recessive disease. However, Bonnycastle *et al*. (8) recently reported a case of autosomal dominant DM without other features of WS. Thus, there is a possibility that genetic heterogeneity of WS exists.

In this study, we report a novel heterozygous mutation of *WFS1* in a WS patient and

demonstrated that this mutant induces constitutive ER stress.

Methods

1) Luciferase assay

1)-1. ERSE luciferase assay

HEK-293 cells were transfected with 1) 1.0 µg of each WT, p.N325_I328del (Del), p.Q194X or p.L543R *WFS1* expression plasmids together with 0.5 µg of ERSE luciferase plasmid (9) and 10 ng *Renilla reniformis* luciferase expression plasmid. Twenty-four hours after transfection, cells were treated with or without 10 nM of thapsigargin for 6 h. The assay was performed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol. ERSE luciferase activity was normalized to *Renilla reniformis* luciferase activity.

1)-2. Cytoplasmic calcium analysis using luciferase assay

HEK-293 cells were transfected with 1.5 µg of each WT, Del, p.Q194X, and p.L543R *WFS1* construct plasmids together with 1.0 µg of nuclear factor of activated T-cell (NFAT) luciferase reporter plasmid (pGL4.30) and 10 ng *Renilla reniformis* luciferase expression plasmids. NFAT is the a transcription factor which is activated by Ca^{2+} sensitive phosphatase calcineurin (10). The cytosolic Ca^{2+} dynamics can be estimated by measuring the NFAT reporter activity (11). Twenty-four hours after transfection, cells were treated with or without 10 nM of thapsigargin for 6 h. The assay was performed as described above.

2) Western blotting

HEK-293 cells were plated on a 10-cm dish and cultured to 70-80% confluency. Cells were transfected with 7.5 µg of each WT or mutant (Del, p.Q194X, p.L543R) FLAG-tagged *WFS1* expression plasmids. Twenty-four hours after transfection, cells were lysed on with ice-cold Mammalian Protein Extraction Buffer (GE Healthcare, Buckinghamshire, UK) containing a protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) for 10 minutes on ice. Then the lysates were centrifuged at 15,000 rpm for 30 minutes at 4 °C. We used NuPAGE LDS Sample Buffer

(Thermo Fisher Scientific, Waltham, MA) for denaturing. Lysates were normalized for to total protein (25 μg per lane) and separated by NuPAGE Novex 4-12% Bis-Tris Protein Gels (Thermo) followed by electroblotting using an iBlot Dry Blotting System (Thermo). Anti-FLAG antibody and anti-β actin antibody were purchased from Sigma Aldrich, respectively. The membrane was blocked with 5% nonfat milk for 1 hour and then added 1:3000 anti-FLAG antibody for overnight at 4°C or 1:3000 anti-β actin antibody for 1 hour at room temperature. Anti-mouse HRP labeled IgG (GE Healthcare) was used as secondary antibody and detected with ECL Western blotting reagents (GE Healthcare).

3) Fluorescence analysis and confocal microscopy

HEK-293 cells were plated on a 25 mm × 75mm plastic chamber slide (Thermo Fisher Scientific, Waltham, MA) and transfected with 3.0 μg WT *WFS1*-GFP or each mutant (Del, p.Q194X, p.L543R) *WFS1*-GFP expression plasmids. Twenty-four hours after transfection, cells were fixed in 4% paraformaldehyde/PBS and permeabilized by adding 0.25% Triton X-100. The ER was stained by an ER-ID® Red Assay Kit (Enzo life Life Science, Farmingdale, NY) according to the manufacturer's protocol. The stained ER and GFP fluorescence was examined on a fluorescence microscope (FLUOVIEW FV1000, Olympus, Tokyo, Japan).

4) Quantitative PCR (qPCR)

HEK-293 cells were seeded into 6-well plates and transfected with 3.0 μg of each WT, Del, p.Q194X, and p.L543R *WFS1* construct plasmids. Twenty-four hours after transfection, mRNA was extracted from 3.0-6.5 × 10^5 cells by NucleoSpin® RNA (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's protocol. RT-PCR was performed with a Veriti® Thermal Cycler using a PrimeScript RT reagent Kit (Takara, Kusatsu, Japan). qPCR was performed with ABI PRISM 7000 (Applied Biosystems, Foster City, CA) and SYBR Premix Ex Taq II (Takara, Kusatsu, Japan) at 95°C for 30 seconds, with 40 cycles at 95°C for 5 seconds and 60°C for 31 seconds. All

reactions were performed in triplicate and the experiments were repeated three times. Values are represented as the mean ± SEM. Statistical significance was tested by one-way ANOVA followed by Tukey's test.

Case report

The patient was born at 41 weeks gestation by normal vaginal delivery as the first child of unrelated Japanese parents. Her birth weight was 3,084 g and her height was 50 cm. Her growth gradually became impaired (Fig. 1). At 6 months of age, bilateral congenital cataracts were noticed. Brain MRI of the patient showed normal bilateral optic nerves. Bilateral congenital cataracts were treated with phacoemulsification and aspiration (PEA) with anterior vitrectom. Her severe hypermetropia (+13 - +15 diopters) was pointed out. As there was no evidence of congenital rubella syndrome, the exact causes of cataracts and hypermetropia were not determined at this time. At 11 months of age, blood tests were carried out and hyperglycemia was detected. At this time, her height was 66.8 cm (−1.8 SD for a normal Japanese girl) and her body weight was 6,050 g (−2.8 SD for a normal Japanese girl). Laboratory examinations were as follows: blood glucose 17.3 mmol/l, HgbA1c 11.6% (103.2 mmol/mol), glycoalbumin 36.4%, serum C-peptide 0.33 nmol/l, and urine C-peptide excretion per day (average of 3 days) 7.6 μg/m^2/day. Anti-glutamic acid decarboxylase and anti-insulinoma-associated 2 antibodies were negative (Table in appendix). These results indicated that her insulin secretion was insufficient, and she was diagnosed with insulin dependent DM. The auditory brainstem response test revealed severe bilateral hearing loss and her development was delayed (roll over at 4 months of age and sit up without support at 10 months of age). Based on the early onset of DM, deafness, cataracts and developmental delays, a diagnosis of WS was suspected. The control of her diabetes remains difficult despite insulin therapy.

Results

WFS1 mutation and subcellular localization of WFS1

Sequence analysis of the patient *WFS1* identified a novel heterozygous twelve base deletion in exon 8 (c.973_984del12). This deletion resulted in an in-frame deletion of four amino acids (p. N325_I328del; Del) (Fig. 2). We analyzed the genomic DNA of the parents and did not find the deletion, indicating that this mutation occurred *de novo*. We also analyzed two hundred normal Japanese individuals, but did not identify this deletion. To determine the intracellular location of the mutant WFS1, we observed the fluorescence of transiently-expressed green fluorescence protein (GFP) fusion WFS1 (WFS1-GFP) in HEK-293 cells. The WFS1 (Del)-GFP was localized in the ER similar to the WT and other mutants (Fig. 3).

Protein expression of the patient WFS1

Western blotting of whole cell lysates showed that the expression levels of mutant WFS1 (Del, p.Q194X and p.L543R) were lower than that of WT. Especially, the expression of p.Q194X could not be detected (Fig. 4).

ERSE luciferase assay

WFS1 plays a role in the suppression of ER stress-mediated cell death by preventing hyperactivation of the ER stress response (9). A previous study showed that two missense mutants (p.H313Y and p.W314R), which were identified in WS and autosomal dominant DM patients respectively, impaired the ability to protect against ER stress (8, 12). In this context, we analyzed the effect of our mutation *in vitro*. Regardless of the presence or absence of the ER stress inducer thapsigargin, our mutant activated the ERSE reporter activity stronger than WT did (Fig. 5A). These results indicate that Del induces constitutive ER stress. Moreover, when WT and Del *WFS1* were equally co-transfected, ER stress was still elevated regardless of the presence or absence of thapsigargin (Fig. 5B). These results indicate that the patient mutant WFS1 causes a dominant negative effect.

Luciferase assay and western blotting of ATF6α

We found that ATF6α promoter activity was elevated in our mutant and that the enhanced signaling was neutralized by a chemical chaperone (4-phenylbutyrate; 4-PBA) (Fig. S1). ATF6α, which localizes to the ER membrane, is a master regulator of the UPR. Under ER stress, the inactive 90-kDa form of ATF6α is cleaved, and the active 50-kDa form of ATF6α is released from the ER (13). Western blotting analysis also showed that Del decreased the expression levels of the inactive form of the 90-kDa ATF6α, but increased the expression level of the active form of ATF6α (50 kDa) (Fig. S2).

Cytosolic Ca^{2+} assay and expression level of SERCA2b and CHOP mRNA

Elevated NFAT reporter activity indicates an elevation of cytoplasmic Ca^{2+} concentration in Del (Fig. 6). The ER is a major source of cytoplasmic Ca^{2+} (14). According to previous studies, WFS1 regulates cellular Ca^{2+} homeostasis by modifying the filling state of the ER Ca^{2+} store by the down regulation of sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) 2b (15, 16). Therefore, we investigated the expression levels of SERCA2b mRNA in WT and Del expressing HEK-293 cells. qPCR showed a decreased mRNA expression level of SERCA2b in Del transfected cells compared with WT transfected cells (Fig. 7(A)). These results suggest that SERCA2b expression levels were decreased in the Del transfected cells, leading to Ca^{2+} efflux from the ER and an elevation of the cytoplasmic Ca^{2+} concentration. The CCAAT-enhancer-binding protein homologous protein (CHOP) mediates ER stress-induced cell apoptosis (17). We revealed that the expression levels of CHOP mRNA were significantly increased in Del expressing HEK-293 cells (Fig. 7(B)). These results suggest that the overexpression of Del leads to ER stress-induced cell apoptosis.

Discussion

In this study, we identified a novel heterozygous mutation of *WFS1* (c.973_984del12, p.N325_I328del) in a patient with WS (Fig. 2). *In vitro* analysis showed that this mutant induced

constitutive ER stress and had a dominant negative effect. Therefore, this mutation is the cause of WS in our patient. Although cataract is not included in the main features of WS, there are several reports of patients who have cataracts. Hansen et al (12) reported five WS families accompanied by congenital cataract. All these cases carried heterozygous or compound heterozygous *WFS1* mutations. In addition, Berry et al. (18) also reported isolated cataracts caused by heterozygous *WFS1* mutation (p.E462G). Interestingly, this case did not have any other features of WS. These findings indicate that cataracts in our patient might be caused by *WFS1* mutation.

Most *WFS1* mutations in WS patients have been detected on both alleles and the inheritance of WS is considered to be autosomal recessive (4). However, a large family of autosomal dominant DM without other features of WS caused by a heterozygous *WFS1* mutation (p.W314R) has been recently reported (8). In this study, eight in thirteen affected members of a family with DM were heterozygous for p.W314R of *WFS1*. *In vitro* studies demonstrated that this mutant lacked the ability to suppress thapsigargin-induced ER stress compared with WT. The authors suggested that the function of WFS1 tetramers composed of p.W314R and WT monomers was impaired, resulting in DM development through a dominant negative effect. Moreover, the other heterozygous *WFS1* mutation (p.H313Y) (12) increases ER stress in the absence of thapsigargin and this constitutive ER stress is caused by ER Ca^{2+} depletion (8, 16). Similar to these results, our mutant also demonstrated constitutive ER stress with a dominant negative effect (Fig. 5(A), 5(B)).

While the expression level of Del was lower than that of WT in western blot, our mutant protein localized to the ER (Fig. 3,4). It is speculated that the mutant may exert the dominant negative effect on the wild type in the ER. Similar to our mutant, the mutant p.H313Y, which is causing constitutive ER stress, also showed the reduced expression level by western blot (16). The discrepancy of reduced expression level and ER localization of our mutant must be further studied.

WFS1 regulates ATF6, which is a key transcription factor involved in UPR signaling through the ubiquitin-proteasome pathway (9). In our experiments, Del increased ATF6α promoter activity compared with WT (Fig. S1). Further western blot analysis showed that activated ATF6α (50 kDa)

was increased in cells transfected with Del (Fig. S2). Normal WFS1 negatively regulates ATF6α, which is one of the UPR signaling molecules involved in the ubiquitin-proteasome pathway. In normal cell conditions, WFS1 recruits ATF6α to the HRD1 (ubiquitin ligase), which marks ATF6α with ubiquitin for proteasome-mediated degradation. Under ER stress conditions, WFS1 releases ATF6α and undergoes proteolysis and the active form of ATF6α moves to the nucleus. Subsequently, ER stress target genes such as CHOP, BIP and XBP-1 are activated (9). ATF6α hyperactivation caused by Del is thought to be one of the causes of WS in this patient. Although the exact mechanism of the increased active form of ATF6α is unknown, the Del-HRD1-ATF6α complex may be unstable.

Because Ca^{2+} signaling is essential for maintaining cellular functions, cytoplasmic and ER Ca^{2+} is tightly regulated. Recently it was reported that WFS1 also acts as a channel regulator by modulating the activity of SERCA (15, 19), a Ca^{2+} modulator localized to the ER membrane, that transports Ca^{2+} from the cytosol into the ER. We found that the cytosolic Ca^{2+} concentrations were elevated in Del overexpressing cells (Fig. 6). Furthermore, the mRNA expression of SERCA2b was decreased in these cells (Fig. 7(A)). These findings indicate that our mutant increases cytosolic Ca^{2+} by reducing the expression level of SERCA2b. Elevated cytosolic Ca^{2+} strongly activates calpain, which is a calcium dependent protease, resulting in β cell apoptosis in WS patients (15, 19). Indeed, in our study, CHOP mRNA expression in Del overexpressing cells was increased (Fig. 7(B)). Therefore, our mutant might induce enhanced β cell apoptosis.

It has been reported that SERCA expression is increased by induction of ER stress (20, 21). In contrast, several studies demonstrated decreased expression levels of SERCA in high glucose or cytokines explored cultured cells, primary islets from db/db mice, human islets from type 2 diabetes patients, and liver from ob/ob mice (16, 22). The discrepancy may be explained by the degree of the strength of ER stress under several pathological conditions. It is speculated that our mutant strongly induces the ER stress beyond the UPR capacity to regulate the SERCA expression.

In conclusion, we identified a novel heterozygous mutation of *WFS1*. Our mutant WFS1 induces constitutive ER stress and cell apoptosis through two pathways: hyperactivation of ATF6 and Ca^{2+} efflux from the ER (Fig. 8). These results provide evidence for new insights into the roles of mutant WFS1 in UPR mechanisms.

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Figure Legends

Fig 1 Growth (height and body weight) charts for the patient in comparison with normal Japanese girls.

Arrow (1), diagnosis of congenital cataract; arrow (2), diagnosis of DM.

Fig 2 PCR-direct sequencing of *WFS1*

(A) Sequence chromatograms of *WFS1* exon 8 in the proband and her parents. Arrow indicates the deletion site. Double bands can be seen after the deletion site. Her parents did not have this mutation, indicative of a *de novo* mutation. (B) Twelve bases are deleted in the patient (c.973_984del12) (shaded box), resulting in an in-frame deletion of four amino acids (p.N325_I328del). (C) Schema of *WFS1*. The bars and black circles show the locations of our mutation (p.N325_I328del) and the mutation that was used in the luciferase assays (p.Q194X, p.L543R). aa; amino acid.

Fig 3 Intracellular localization of WFS1 protein.

HEK-293 cells were transfected with WT *WFS1*-GFP or mutant (p.N325_I328del, p.Q194X, p.L543R)-GFP expression plasmids. The location of the ER was determined by ER-ID® Red Assay Kit (Enzo life science, NY). WT, wild type; Del, p.N325_I328del; 194, p.Q194X; 543, p.L543R.

Fig 4 Western blotting with protein extracted from HEK-293 cells.

HEK-293 cells were transfected with FLAG tagged WT or mutant (p.N325_I328del, p.Q194X, p.L543R) *WFS1* expression plasmids. Protein was extracted and protein expression levels were analyzed by western blotting using anti-FLAG and anti-β actin antibodies. The expression levels of mutant WFS1 (p.N325_I328del, p.Q194X, p.L543R) were lower than that of WT.

WT, wild type; Del, p.N325_I328del; 194, p.Q194X; 543, p.L543R.

Fig 5 Luciferase reporter assays in HEK-293 cells.

(A) HEK-293 cells were transfected with the ERSE reporter vector together with control (pcDNA3), WT *WFS1*, and mutant (p.N325_I328del, p.Q194X, p.L543R) expression plasmids (n = 9 for each). Cells were treated with or without 10 nM thapsigargin for 6 h and the luciferase intensity was measured. Data were normalized to *Renilla reniformis* luciferase activity. All values are represented as the mean ± SEM. Statistical significance was tested by one-way ANOVA followed by Tukey's test. *, $P < 0.001$; **, $P = 0.024$; ***, $P = 0.029$.

ER stress caused by thapsigargin was unremarkable in WT cells, but was prominent in pcDNA3, p.N325_I328del, and p.L543R transfected cells. Furthermore, p.N325_I328del yields ER stress regardless of the presence or absence of thapsigargin.

(B) HEK-293 cells were transfected with the ERSE reporter vector simultaneously in WT *WFS1* alone or WT together with the mutant (p.N325_I328del) expression plasmids (n = 9 for each). Cells were treated with or without 10 nM thapsigargin for 6 h and the luciferase intensity was measured. Data were normalized to *Renilla reniformis* luciferase activity. All values are represented as the mean ± SEM. Statistical significance was tested by one-way ANOVA followed by Tukey's test. *, $P < 0.0001$; **, $P < 0.001$.

ER stress was elevated by thapsigargin when WT and p.N325_I328del *WFS1* were equally co-transfected. Moreover, ER stress is constitutively elevated regardless of the presence or absence of thapsigargin. These results indicate that our mutant p.N325_I328del causes a dominant negative effect.

TG, thapsigargin; WT, wild type; Del, p.N325_I328del; 194, p.Q194X; 543, p.L543R.

Fig 6 Luciferase reporter assay using expression plasmid with an NFAT response element (pGL4.30, Promega).

HEK-293 cells were co-transfected with pGL4.30 together and WT *WFS1* or mutant

(p.N325_I328del, p.Q194X, p.L543R) expression plasmids. Cells were treated with or without 10 nM thapsigargin for 6 h and the luciferase intensity was measured. Data were normalized to *Renilla reniformis* luciferase activity. All values are represented as the mean ± SEM. Statistical significance was tested by one-way ANOVA followed by Tukey's test. *, $P < 0.001$; **, $P < 0.01$; *** $P = 0.0454$.

NFAT response reporter activity of our mutant was higher than that of WT regardless of the presence or absence of thapsigargin.

WT, wild type; Del, p.N325_I328del; 194, p.Q194X; 543, p.L543R.

Fig 7 Quantitative PCR analysis of SERCA2b and CHOP mRNA expression.

(A) **Quantitative PCR analysis of SERCA2b**; HEK-293 cells were transfected with WT *WFS1* or p.N325_I328del expression plasmids. mRNA was extracted from cells and RT-PCR was performed followed by quantitative PCR. All reactions were performed in triplicate and the experiment was repeated two times. The values are represented as the mean ± SEM. Statistical significance was tested by one-way ANOVA followed by Tukey's test. *, $P < 0.01$; **, $P = 0.0214$.

The expression level of SERCA2b mRNA in p.N325_I328del transfected cells was decreased compared with WT.

(B) **Quantitative PCR analysis of CHOP**; HEK-293 cells were transfected with WT *WFS1* or mutant (p.N325_I328del, p.Q194X, p.L543R) expression plasmids. mRNA was extracted from the cells and RT-PCR was performed followed by quantitative PCR. All reactions were performed in triplicate and the experiment was repeated three times. The values are represented as the mean ± SEM. Statistical significance was tested by one-way ANOVA followed by Tukey's test. *, $P < 0.001$; **, $P < 0.01$; ***, $P = 0.0122$.

The expression levels of CHOP mRNA were significantly increased in p.N325_I328del expressing HEK-293 cells.

WT, wild type; Del, p.N325_I328del; 194, p.Q194X; 543, p.L543R.

Fig 8 Schematic of the pathogenesis of mutant WFS1 (p.N325_I328del).

The mutant WFS1 increases cytosolic Ca^{2+} by reducing the expression level of SERCA2b. Simultaneously, the mutant WFS1 causes constitutive ATF6α hyperactivation. These two pathways activate the apoptotic effector of UPR and mediate the pathogenesis of severe Wolfram syndrome.

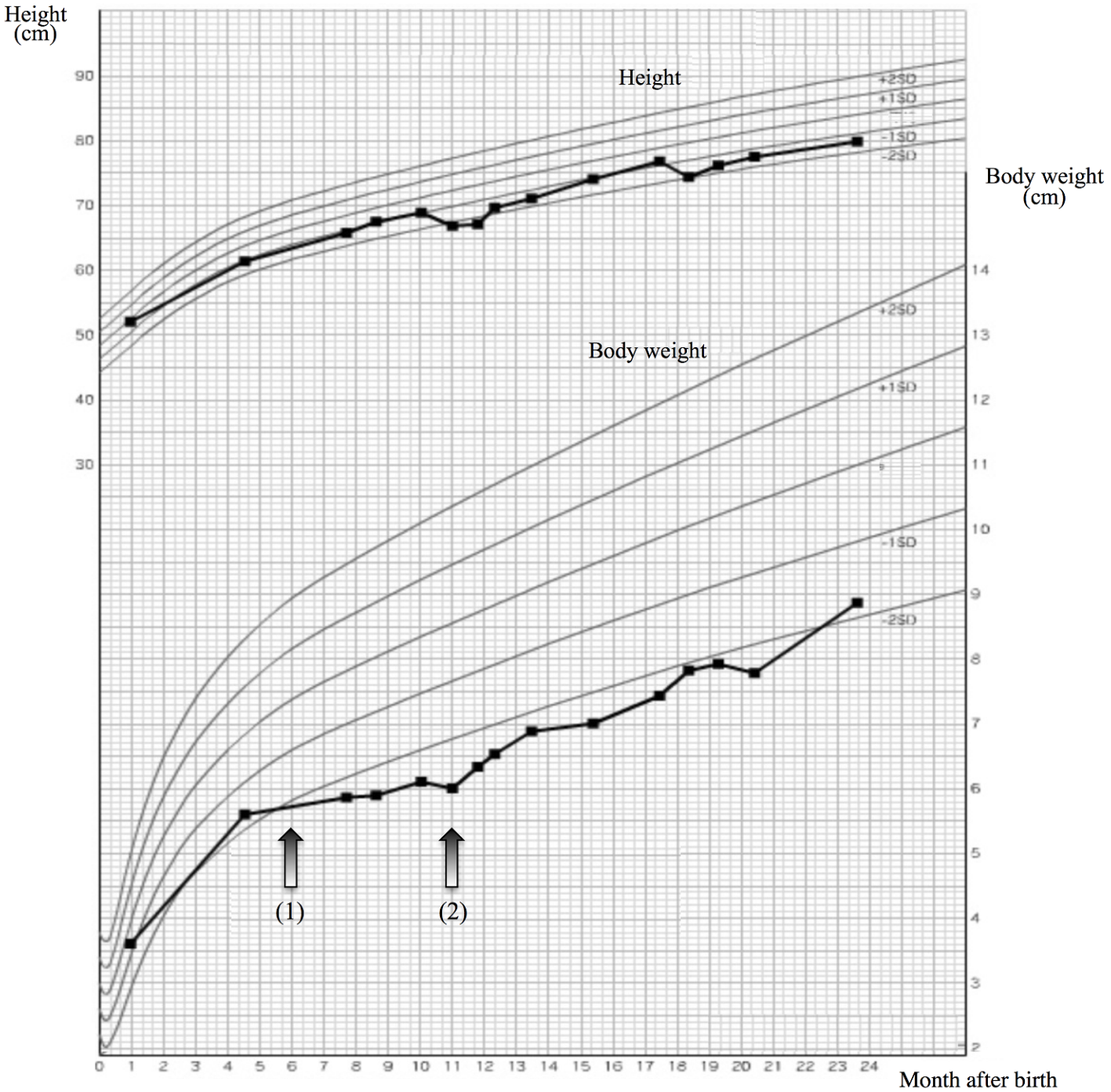
Height
(cm)
Height
+2SD
+1SD
-1SD
-2SD
Body weight
(cm)
Body weight
+2SD
+1SD
-1SD
-2SD
(1)
(2)
Month after birth

Fig. 1

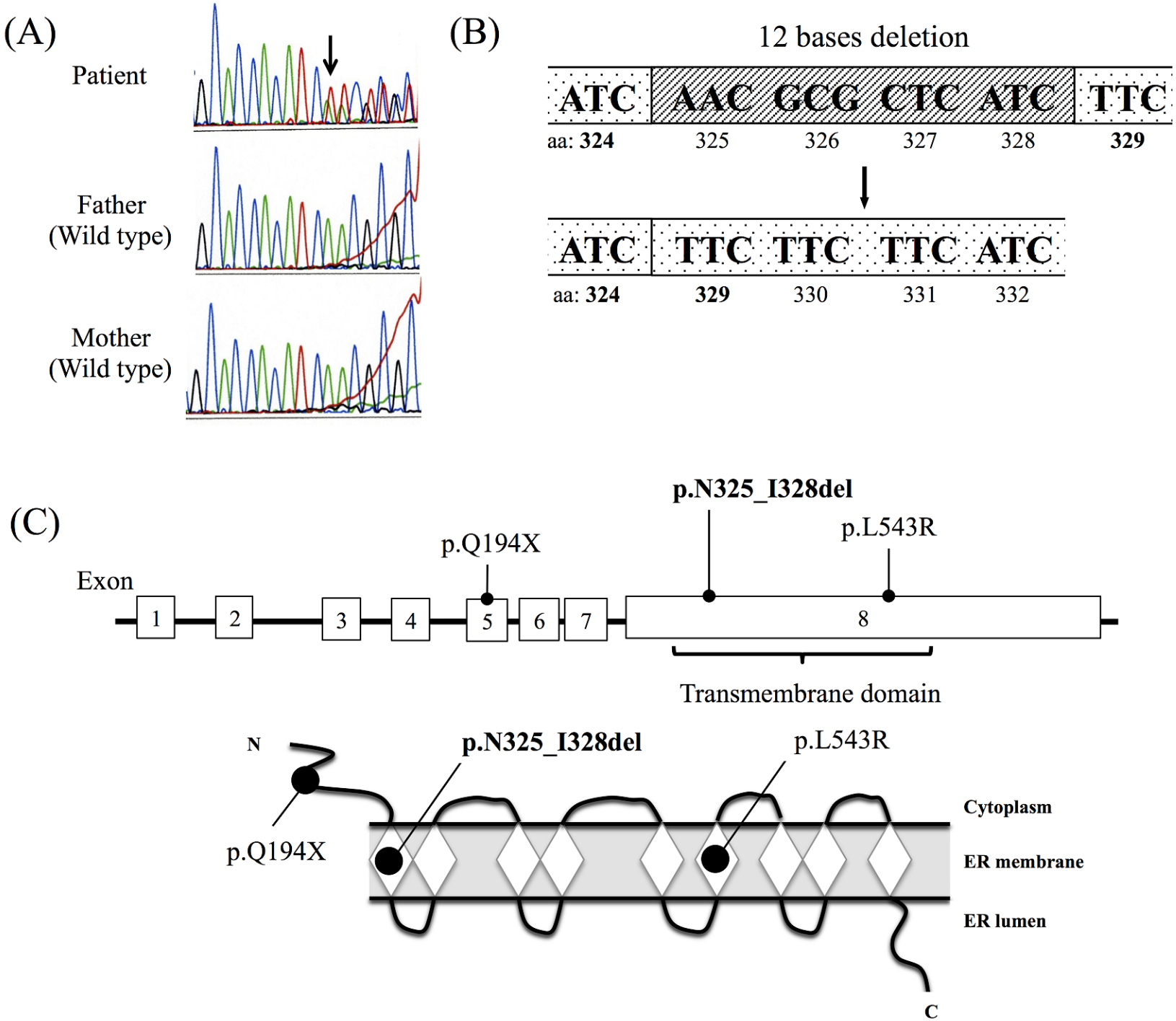
(A)
Patient
Father
(Wild type)
Mother
(Wild type)
(B)
12 bases deletion
ATC AAC GCG CTC ATC TTC
aa: 324 325 326 327 328 329
ATC TTC TTC TTC ATC
aa: 324 329 330 331 332
(C)
Exon
1 2 3 4 5 6 7 8
p.Q194X
p.N325_I328del
p.L543R
Transmembrane domain
N
p.N325_I328del
p.L543R
p.Q194X
Cytoplasm
ER membrane
ER lumen
C

Fig. 2

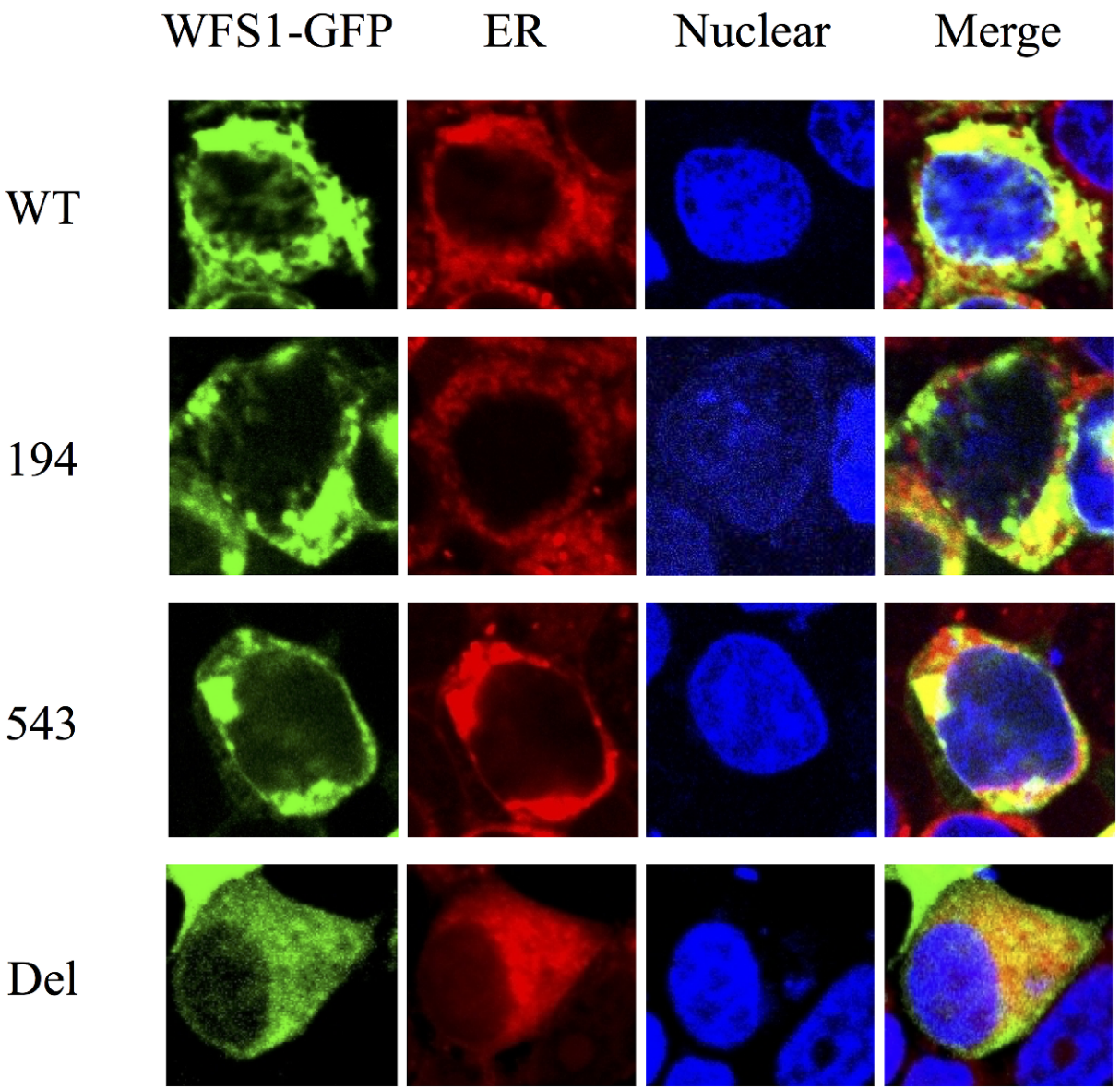

Fig. 3

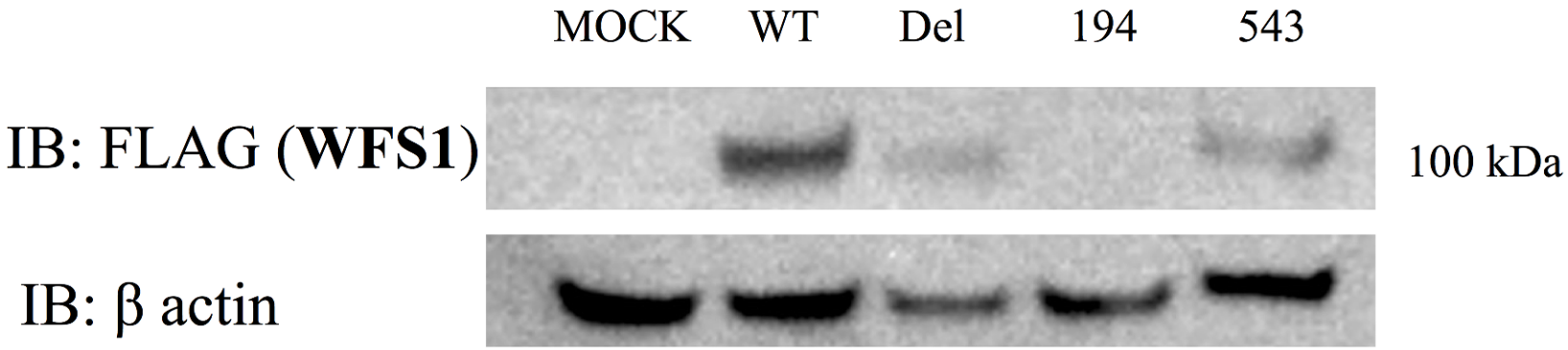

Fig. 4

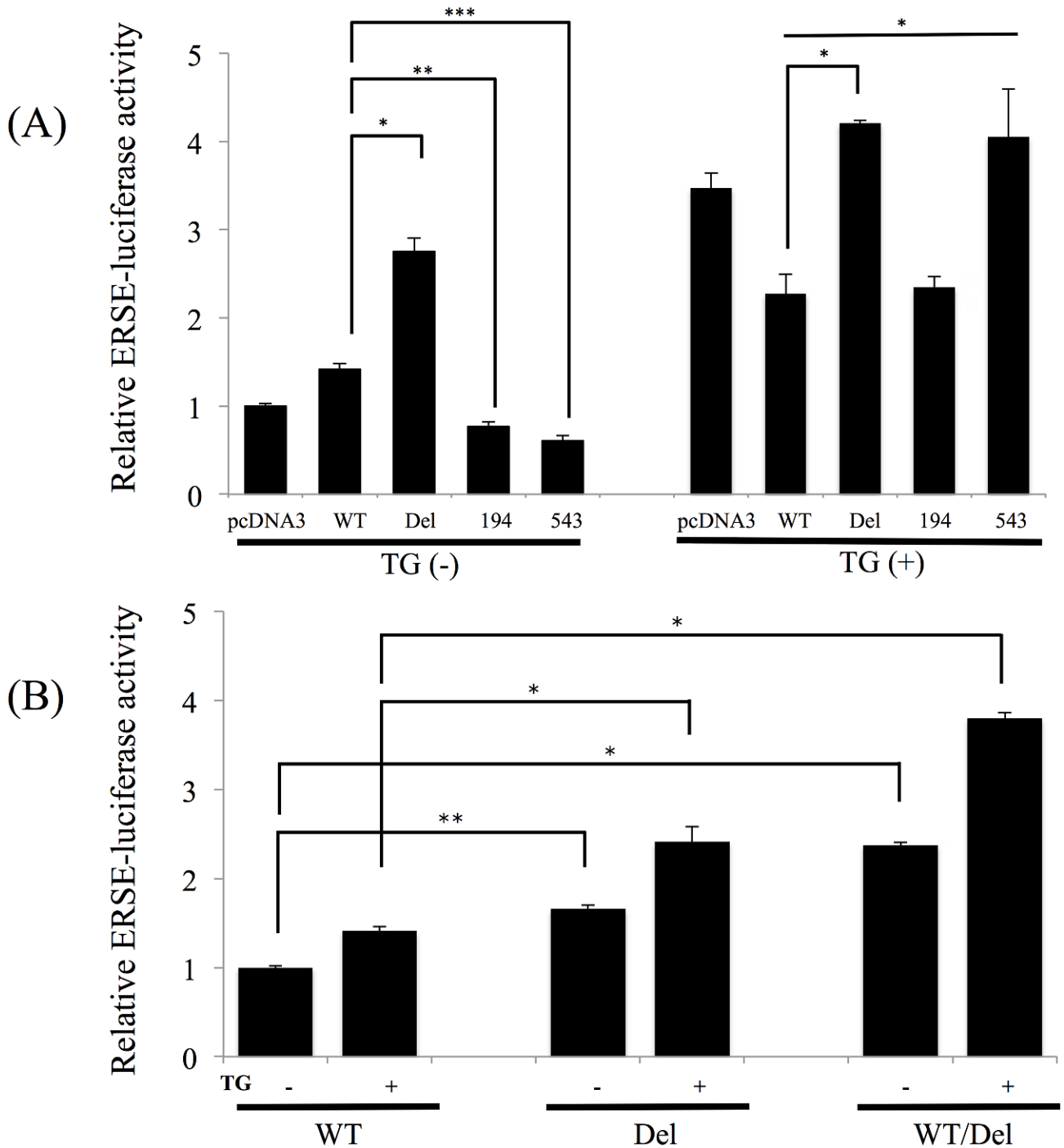
(A)
Relative ERSE-luciferase activity
0
1
2
3
4
5
***
**
*
*
*
pcDNA3
WT
Del
194
543
pcDNA3
WT
Del
194
543
TG (-)
TG (+)
(B)
Relative ERSE-luciferase activity
0
1
2
3
4
5
*
*
*
**
TG
-
+
-
+
-
+
WT
Del
WT/Del

Fig. 5

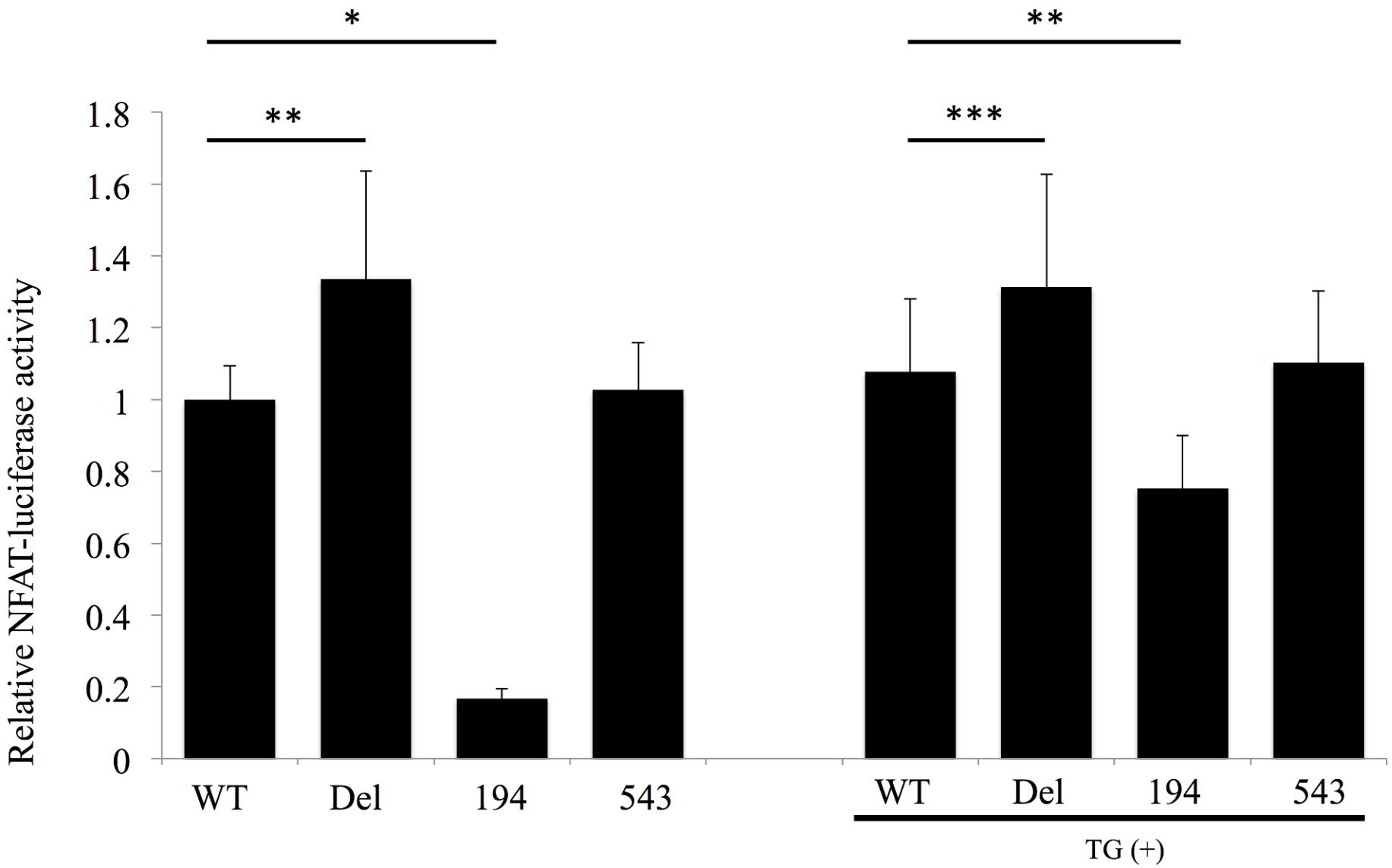
*
**
**
***
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative NFAT-luciferase activity
WT
Del
194
543
WT
Del
194
543
TG (+)

Fig. 6

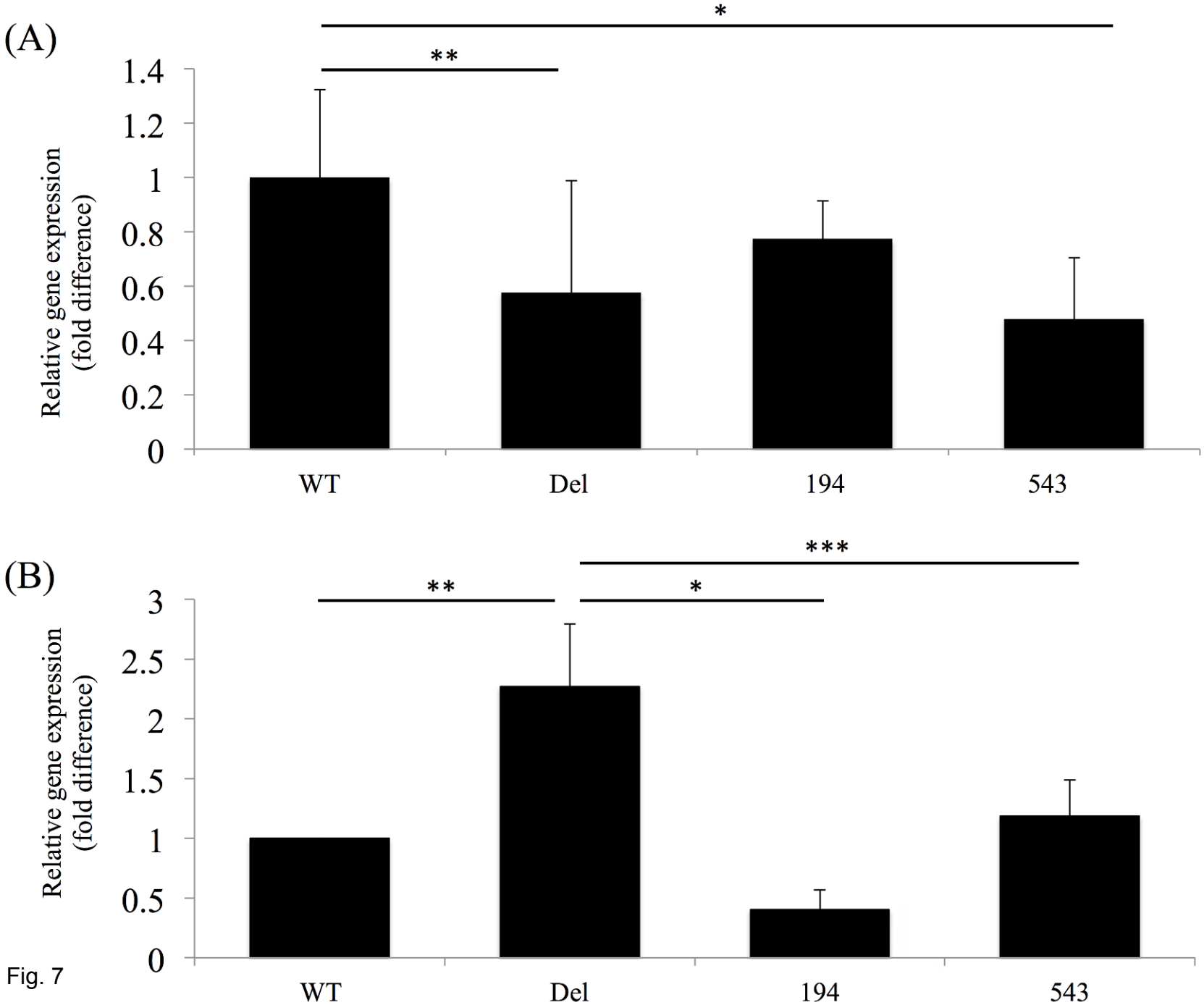
(A)
*
**
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Relative gene expression
(fold difference)
WT
Del
194
543
(B)
***
**
*
3
2.5
2
1.5
1
0.5
0
Relative gene expression
(fold difference)
WT
Del
194
543

Fig. 7

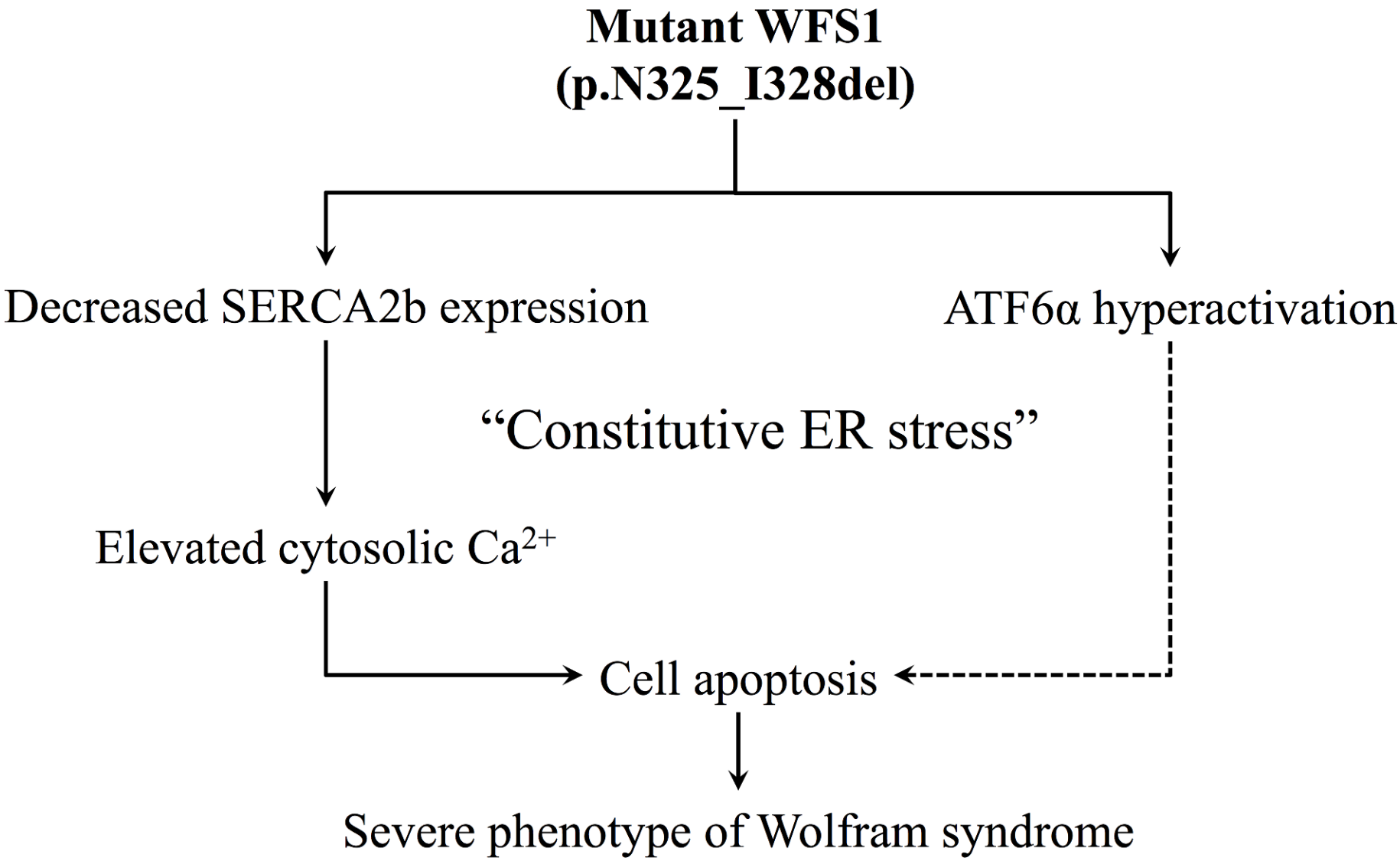
Mutant WFS1
(p.N325_I328del)
Decreased SERCA2b expression
ATF6α hyperactivation
"Constitutive ER stress"
Elevated cytosolic Ca^{2+}
Cell apoptosis
Severe phenotype of Wolfram syndrome

Fig. 8