indicated antibodies. Data shown are representative of at least three experiments with different sets of samples.

B) Expressions of chaperone proteins in MIN6wfs1$^{-/-}$ cells. (Upper panel) Cellular lysates were probed with anti-GRP78, anti-KDEL and anti-actin (loading control) antibodies. (Lower panel) MIN6wfs1$^{+/-}$ (open columns) and MIN6wfs1$^{-/-}$ (closed columns) cells were transiently transfected with the pGL3-promoter plasmid containing the SV40 promoter-luciferase (SV40pro: 0.5 μg) or pGRP78pro(-172)-Luc (GRP78pro: 0.5 μg) together with the reference plasmid pTK-RL (0.05 μg) encoding *Renilla* luciferase. Twenty-four hours after transfecion, cellular lysates were subjected to luciferase assay. The luciferase activity of the pGL3-promoter in MIN6wfs1$^{+/-}$ was defined as 1. The averages of three independent experiments, each performed in duplicate, are presented. *$p < 0.05$, $n = 3$.

C) Suppression of PERK phosphorylation by WFS1 re-expression in MIN6wfs1$^{-/-}$ cells. Cells were infected with AdCAG-TR expressing Tet-repressor (TR) and AdCTO-WFS1 harboring *WFS1* cDNA. WFS1 expression was induced by 48 hour doxycycline (DOX, 2 μg/ml) treatment. The experiment was repeated three times and similar results were obtained.

C) Suppression of PERK phosphorylation by GRP78 overexpression in MIN6wfs1$^{-/-}$ cells. Human GRP78 expression was induced by 48 hour DOX treatment. The experiment was repeated four times and similar results were obtained.

Figure 3. No UPR changes in heart, skeletal muscle or brown adipose tissue from *wfs1*-deficient mice.

A) WFS1 protein distribution in mice. Approximately 100 μg of protein from wild-type

mouse tissues were analyzed for the presence of WFS1 protein. BAT, brown adipose tissue; WAT, white adipose tissue.

B - D) UPR activation was not observed in heart (B), skeletal muscle (C) or BAT from *wfs1*-deficient mice. The Western blot data shown are representative of two experiments, each performed using three mice of each genotype.

Figure 4. Activation of apoptosis signaling in *wfs1*-deficient islets and MIN6 cells.

A) Real-time RT-PCR analysis of CHOP mRNA in wild-type (open column) and *wfs1*-deficient (closed column) islets. Relative mRNA levels were obtained after normalization to actin mRNA. *$p < 0.05$, $n = 6$.

B) Western blot analysis of apoptosis signaling proteins in *wfs1*-deficient islets. Lysates of islets were probed with the indicated antibodies: P-JNK, phospho-JNK; T-JNK, total-JNK. Data shown are representative of three experiments with different sets of samples.

C) Increased expression of CHOP and cleaved caspase-3 in *wfs1*-deficient MIN6 cells. Lysates of MIN6 cell derivatives were probed with the indicated antibodies. Data shown are representative of three experiments.

Figure 5. Impaired cell cycle progression and increased $p21^{CIP1}$ expression in *wfs1*-deficient islets.

A, B) Impaired cell cycle progression in *wfs1*-deficient β-cells. Incorporated BrdU and insulin were probed with specific antibodies (A) and BrdU positive β-cells were counted (B). Bars, 10 μm. *$p < 0.05$, $n = 4$ mice per group.

C, D) Increased $p21^{CIP1}$ expression in *wfs1*-deficient islets and MIN6 cells. Lysates of

wild-type and *wfs1*-deficient islets (C) or MIN6 cells (D) were probed with the indicated antibodies: T-p53, total-p53; P-p53, phospho-p53. Data shown are representative of three experiments with different sets of samples.

E, F) Induction of $p21^{CIP1}$ expression by thapsigargin (TG) in islets (E) and MIN6 cells (F). Wild-type islets were challenged with 0.5 μM TG for 12 h. MIN6 cells were also treated with 0.5 μM TG for the indicated durations. Lysates of islets or MIN6 cells were probed with the indicated antibodies. The experiment was repeated three times and similar results were obtained.

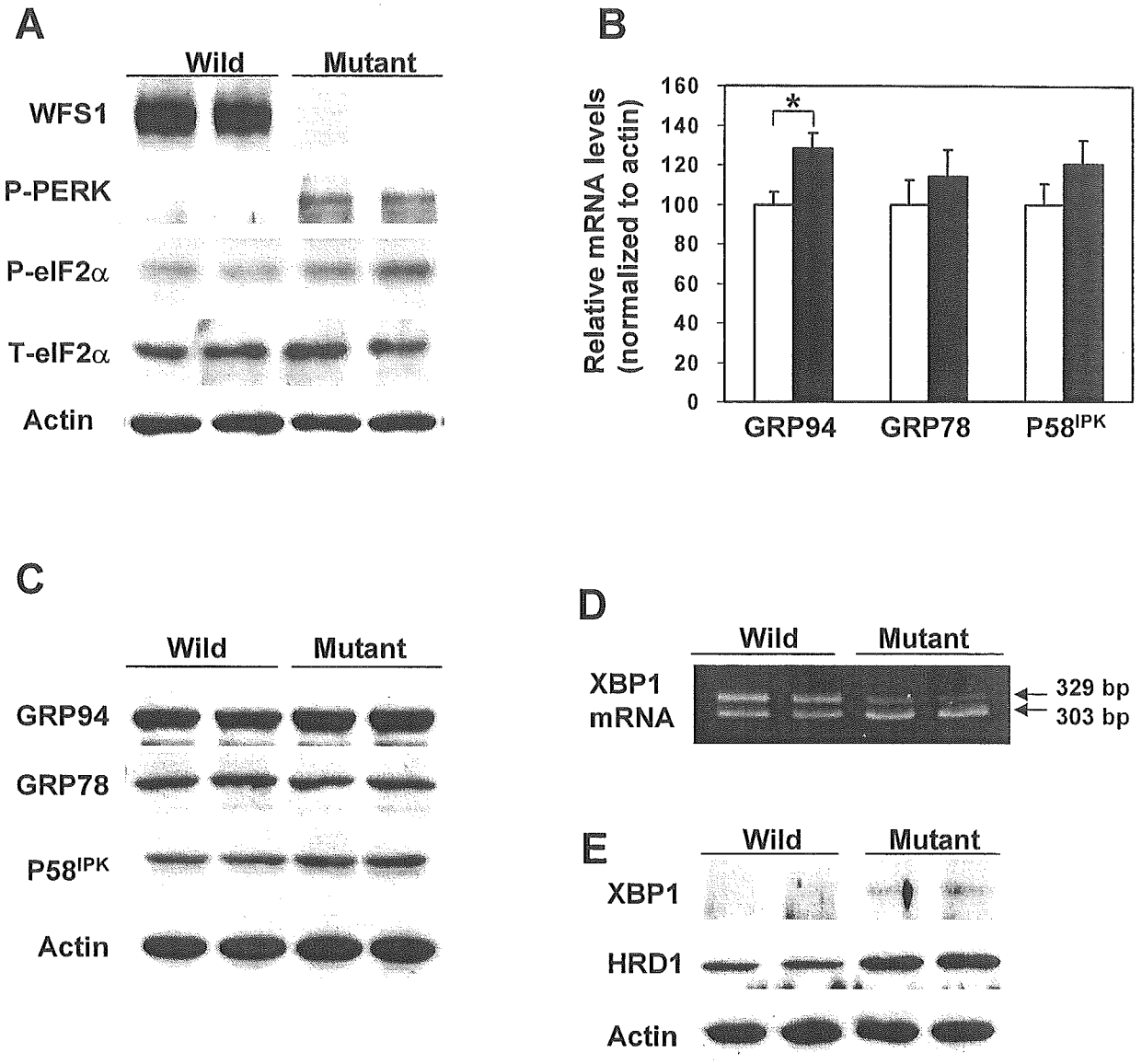
Figure 6. Decrease in MIN6 cell numbers in response to forced $p21^{CIP1}$ expression.

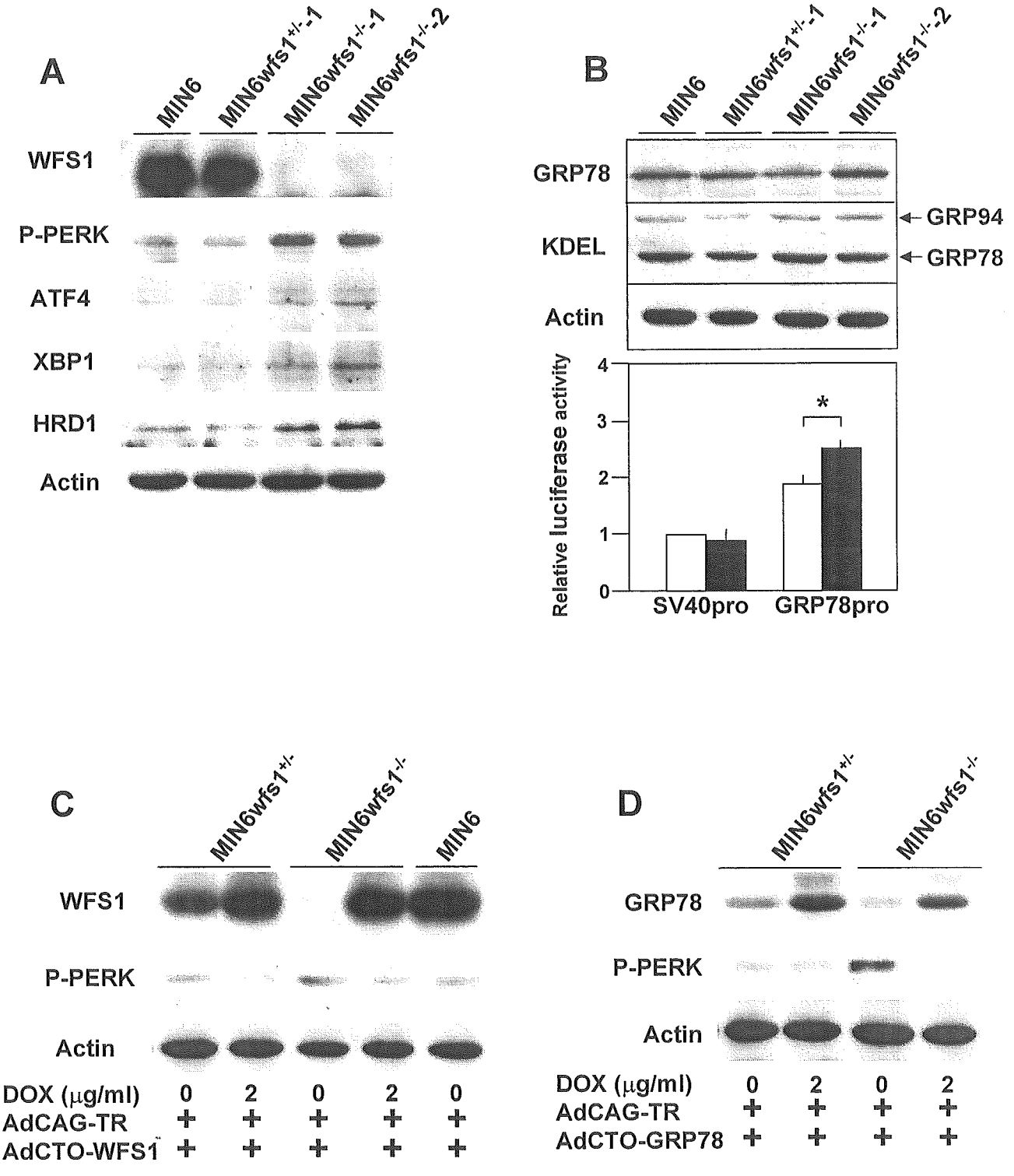
A) Forced expression of $p21^{CIP1}$ in MIN6 cells. Cells were either uninfected or infected with AdCAG-TR (m.o.i. of 30) and AdCTO-$p21^{CIP1}$ (m.o.i. of 100) harboring $p21^{CIP1}$ cDNA. Expression of $p21^{CIP1}$ was induced by 48 hour DOX (2 μg/ml) treatment. MIN6 cell lysates were subjected to immunoblot analysis using anti-$p21^{CIP1}$ and actin antibodies.

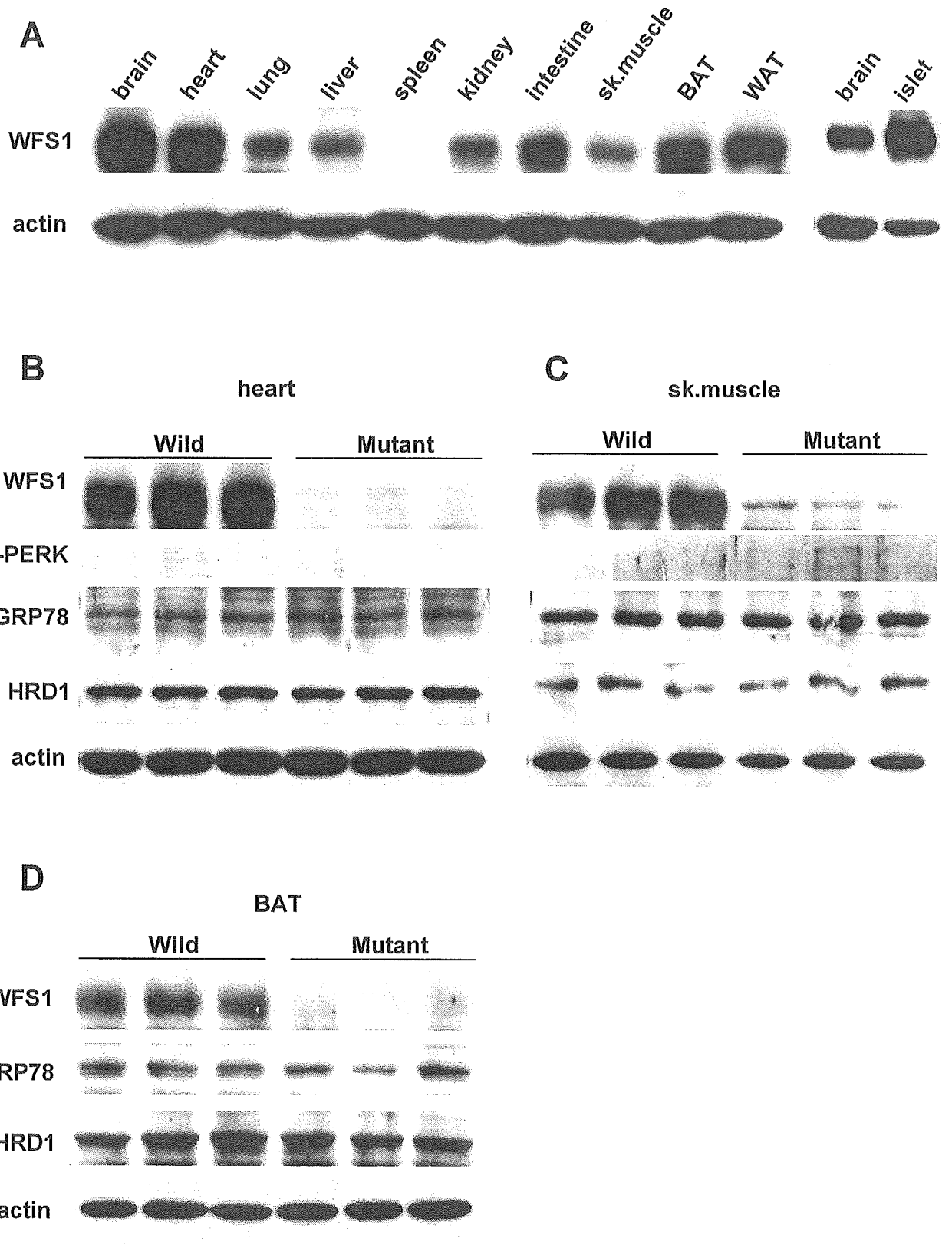
B) Numbers of MIN6 cells overexpressing $p21^{CIP1}$. One day after adenovirus transduction, cells were reseeded (2×10^5 per well) and divided into two groups, and, after two more days, treatment with (closed circles) or without (open circles) DOX (2 μg/ml) was commenced (day 0). Uninfected MIN6 cells (open squares) were also seeded two days before. Cells were then harvested on days 0, 2, 4 and 6, stained with trypan blue, and counted. Data are means ± S.E. for triplicate wells. $^{**}p < 0.01$ against both controls. The experiment was repeated three times and similar results were obtained.

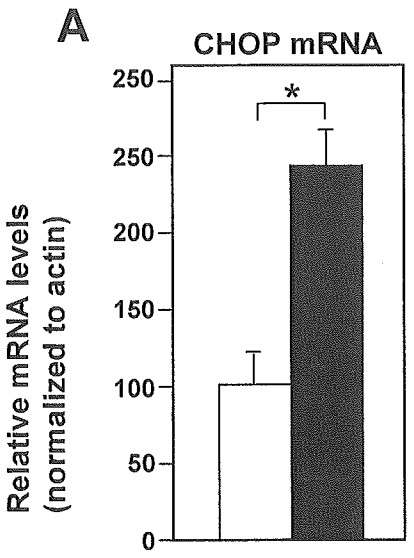
Table 1. Primers used for quantitative real-time RT-PCR

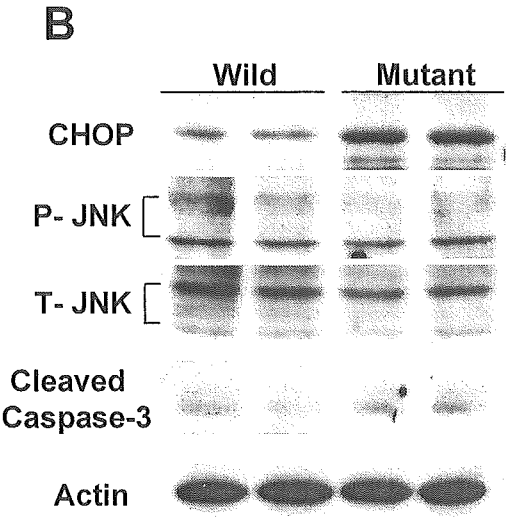
Genes	Forward	Reverse
ATF4	5'-TCCTGAACAGCGAAGTGTTG-3'	5'-ACCCATGAGGTTTCAAGTGC-3'
GRP94	5'-TGATGAAGTCGACGTGGATG-3'	5'-TCCTGTTCACTTCAGCTTGG-3'
GRP78	5'-GACATTTGCCCCAGAAGAAA-3'	5'-CTCATGACATTCAGTCCAGCA-3'
$P58^{IPK}$	5'-CCTTATCGGACAGTCCTTCG-3'	5'-TCAGAGTCCTGATTTCATCTTCA-3'
EDEM	5'-GGAAATTCATCCGAGTTCCA-3'	5'-GGGCCATGTACAACAATTCA-3'
CHOP	5'-CCTAGCTTGGCTGACAGAGG-3'	5'-CTGCTCCTTCTCCTTCATGC-3'
GADD34	5'-CGGAGAGAAGCCAGAATCAC-3'	5'-CAGCAAGGAAATGGACTGTG-3'
$P21^{CIP1}$	5'-ACATCTCAGGGCCGAAAAC-3'	5'-CCTGACCCACAGCAGAAGAG-3'

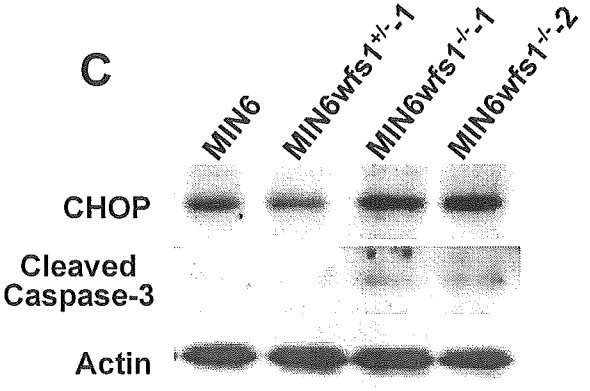

A
Wild
Mutant
WFS1
P-PERK
P-eIF2α
T-eIF2α
Actin
B
Relative mRNA levels (normalized to actin)
160
140
120
100
80
60
40
20
0
*
GRP94
GRP78
P58IPK
C
Wild
Mutant
GRP94
GRP78
P58IPK
Actin
D
Wild
Mutant
XBP1 mRNA
329 bp
303 bp
E
Wild
Mutant
XBP1
HRD1
Actin

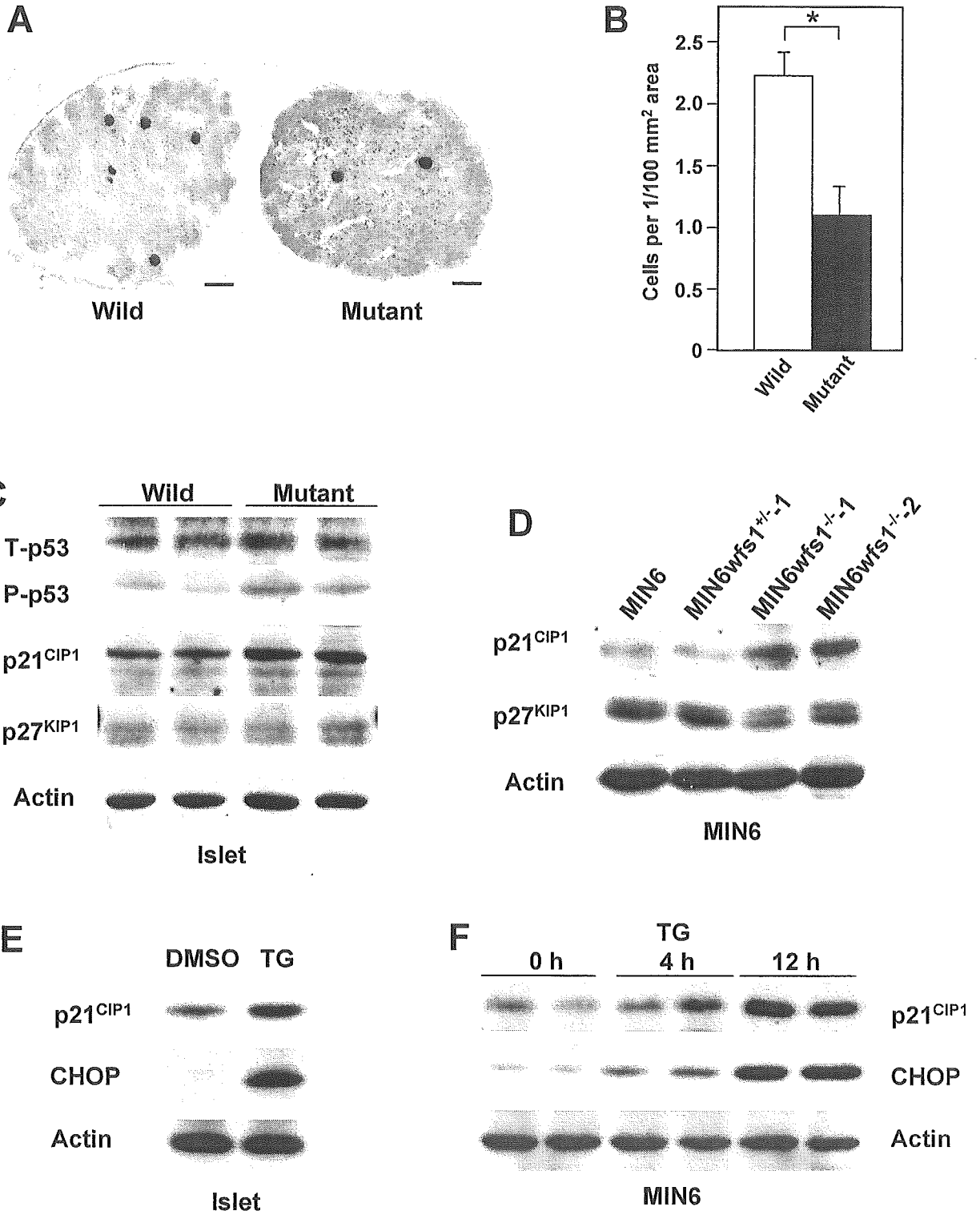

A
MIN6
MIN6wfs1+/--1
MIN6wfs1-/--1
MIN6wfs1-/--2
WFS1
P-PERK
ATF4
XBP1
HRD1
Actin
B
MIN6
MIN6wfs1+/--1
MIN6wfs1-/--1
MIN6wfs1-/--2
GRP78
KDEL
Actin
GRP94
GRP78
Relative luciferase activity
0
1
2
3
4
*
SV40pro
GRP78pro
C
MIN6wfs1+/-
MIN6wfs1-/-
MIN6
WFS1
P-PERK
Actin
DOX (μg/ml) 0 2 0 2 0
AdCAG-TR + + + + +
AdCTO-WFS1 + + + + +
D
MIN6wfs1+/-
MIN6wfs1-/-
GRP78
P-PERK
Actin
DOX (μg/ml) 0 2 0 2
AdCAG-TR + + + +
AdCTO-GRP78 + + + +

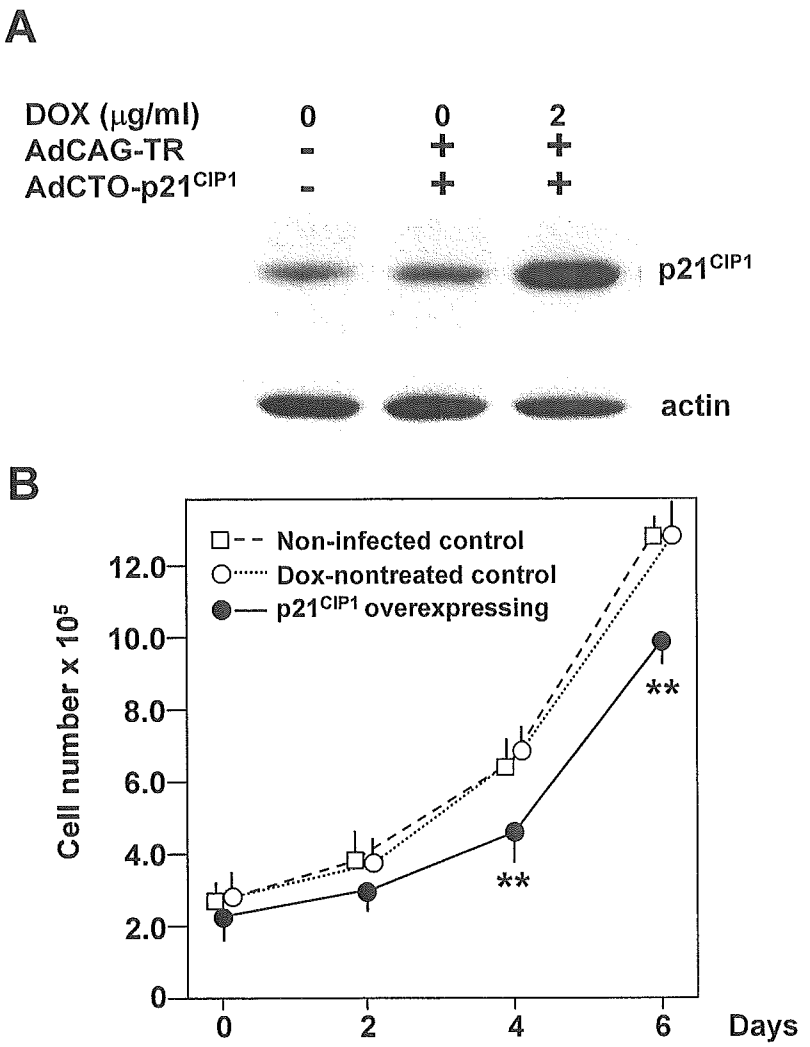

A
brain
heart
lung
liver
spleen
kidney
intestine
sk.muscle
BAT
WAT
brain
islet
WFS1
actin
B
heart
Wild
Mutant
WFS1
P-PERK
GRP78
HRD1
actin
C
sk.muscle
Wild
Mutant
D
BAT
Wild
Mutant
WFS1
GRP78
HRD1
actin


A
CHOP mRNA
Relative mRNA levels
(normalized to actin)
250
250
200
150
100
50
0
*


B
Wild
Mutant
CHOP
P- JNK
T- JNK
Cleaved
Caspase-3
Actin


C
MIN6
MIN6wfs1+/- -1
MIN6wfs1-/- -1
MIN6wfs1-/- -2
CHOP
Cleaved
Caspase-3
Actin


A
Wild
Mutant
B
Cells per 1/100 mm² area
2.5
2.0
1.5
1.0
0.5
0
*
Wild
Mutant
C
Wild
Mutant
T-p53
P-p53
p21CIP1
p27KIP1
Actin
Islet
D
MIN6
MIN6wfs1+/--1
MIN6wfs1-/--1
MIN6wfs1-/--2
p21CIP1
p27KIP1
Actin
MIN6
E
DMSO
TG
p21CIP1
CHOP
Actin
Islet
F
TG
0 h
4 h
12 h
p21CIP1
CHOP
Actin
MIN6


A
DOX (μg/ml) 0 0 2
AdCAG-TR - + +
AdCTO-p21CIP1 - + +
p21CIP1
actin
B
Non-infected control
Dox-nontreated control
p21CIP1 overexpressing
Cell number x 10^5
12.0
10.0
8.0
6.0
4.0
2.0
0
0
2
4
6
Days
**
**

Abbreviations

BrdU, 5-bromodeoxyuridine; DOX, doxycycline; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; ERAD, ER-associated degradation; PERK, RNA-dependent protein kinase-like ER kinase; UPR, unfolded protein response.

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Brief report

A novel mutation of *WFS1* gene in a Japanese man of Wolfram syndrome with positive diabetes-related antibodies

Akinobu Nakamura [a], Chikara Shimizu [a,*], So Nagai [a], Satoshi Taniguchi [a], Masaaki Umetsu [a], Toshiya Atsumi [a], Norio Wada [a], Narihito Yoshioka [a], Yuri Ono [a], Yukio Tanizawa [b], Takao Koike [a]

[a] *Department of Medicine II, Hokkaido University Graduate School of Medicine, N-15, W-7, Kita-ku, Sapporo 060-8638, Japan*
[b] *Division of Molecular Analysis of Human Disorders, Department of Bio-Signal Analysis, Yamaguchi University Graduate School of Medicine, Minamikogushi 1-1-1, Ube 755-8505, Japan*

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Abstract

Wolfram syndrome is a rare, autosomal recessive disorder characterized by early-onset diabetes mellitus, optic atrophy and neurological and endocrinological abnormalities. A 47-year-old Japanese man with frequent severe hypoglycemic episodes was diagnosed as Wolfram syndrome based on clinical features and laboratory data. He had positive glutamic acid decarboxylase (GAD) and insulinoma-associated antigen-2 (IA-2) antibodies, both uncommon in this syndrome. Genetic analysis revealed that *WFS1* gene of the patient has a homozygous 5 base pairs (AAGGC) insertion at position 1279 in exon 8, causing a frameshift at codon 371 leading to premature termination at codon 443.

Keywords: Wolfram syndrome; GAD antibody; IA-2 antibody; *WFS1* gene

Wolfram syndrome (WS), also referred to as DIDMOAD syndrome, is an autosomal recessively inherited syndrome first described by Wolfram and Wagener [1]. It is a progressive neurodegenerative syndrome characterized by diabetes insipidus, diabetes mellitus, optic atrophy and deafness. WS is rare with an estimated general prevalence of 1:770,000 and a carrier frequency of 1:354 [2]. In 1998, a nuclear gene responsible for WS, *WFS1*, was identified and mapped to chromosome 4p16.1 using positional cloning [3].

The following report is of a patient diagnosed with WS having positive glutamic acid decarboxylase (GAD) and insulinoma-associated antigen-2 (IA-2) antibodies. The diabetogenic mechanism in this syndrome has apparently not been recognized as an autoimmune process. Furthermore, we identified a novel mutation in the *WFS1* gene of the patient.

A 47-year-old Japanese man was admitted to our hospital because of frequent severe hypoglycemic episodes. Diabetes was diagnosed and insulin treatment was started when he was 6 years. Progressive loss of vision was observed at the age of 11 and hearing loss at 19 years. At the age of 24 years, he was aware of difficulty in urinating, which required daily bladder catheterizations. His parents, who are healthy as is the older brother, are consanguineous. No hereditary disease in the ascendants was found.

On physical examination, height was 165.1 cm and weight was 37.3 kg. Blood pressure was 108/62 mmHg,

* Corresponding author. Tel.: +81 11 706 5915; fax: +81 11 706 7710.
E-mail address: shimizch@med.hokudai.ac.jp (C. Shimizu).

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Table 1
Diabetes-related findings

Fasting plasma glucose (mg/dl)	109
Glycohemoglobin A_{1c} (HbA_{1c}) (%)	9.2
Urinary output of C peptide reactivity (CPR) (μg/day)	<0.7
Plasma CPR to glucagon (0–6 min) (ng/ml)	<0.05 to <0.05
Urinary microalbumin (mg/day)	3.2
Glutamic acid decarboxylase (GAD) antibody	(+)
Insulinoma-associated antigen-2 (IA-2) antibody	(+)
Autoantibodies to iscet cells (ICA)	(−)
Human leucocyte antigen (HLA)	DR-4, DR-8
Mitochondrial tRNA Leu (3243) mutation	(−)

and pulse rate was regular at 66 min^{-1}. There were no remarkable findings in heart, lung and abdomen. Neurological abnormalities such as cerebellar ataxia and myoclonus were not found except for the absence of Achilles tendon reflex. Psychiatric disorders such as depression and psychosis were not found.

In general laboratory findings, urine sugar was detected. Other general biochemical markers including renal function, electrolytes and lipid profiles were within normal range as were serum concentrations of lactate and pyruvate.

Diabetes-related findings are shown in Table 1. Diabetic neuropathy was found, but diabetic nephropathy and diabetic retinopathy were not. A high level of glycohemoglobin A_{1c} (HbA_{1c}), an extremely low level of urinary output of C peptide reactivity (CPR) and unresponsiveness of CPR to glucagon loading test were comparable with insulin-dependent diabetes mellitus commonly seen in patients with WS. However, GAD and IA-2 antibodies were detected, being uncommon in WS. Autoantibodies to islet cells were negative. Human leucocyte antigen (HLA) typing of DR was DR-4 and DR-8. The mitochondrial tRNA Leu (3243) mutation was absent.

Optic atrophy was confirmed by fundascopy and a bilateral symmetric sensorineural hearing loss prevalent for the medium–high frequencies was demonstrated by audiometry. Urodynamics testings showed bladder atony regardless of no upper urinary tract abnormalities. Brain magnetic resonance imaging (MRI) showed the absence of posthypophysis signals, but other MRI abnormalities such as cerebellar and brain stem atrophy were not found. To confirm the presence of diabetes insipidus (DI), hypertonic saline test was done. Arginine vasopressin (AVP) was not responsive to 5% hypertonic saline infusion (from 1.48 pmol/l to 1.48 pmol/l), which confirmed the diagnosis of DI.

Genetic analysis was made under the approval of the institutional review board and written informed consent. Using genomic DNA extracted from peripheral blood mononuclear cells, all exons of *WFS1* gene were amplified by polymerase-chain-reaction (PCR) and directly sequenced, as described in a previous report [3]. The patient has a homozygous 5 base pairs (AAGGC) insertion at position 1279 in exon 8 which causes a frameshift at codon 371 leading to premature termination at codon 443. Family analysis demonstrated that his parents but not his brother are heterozygous for the same mutation as the patient. Family members other than the patient did not show any signs suggesting WS.

There are several clear distinctions between WS-associated diabetes and classic type 1 diabetes. It is well established that genes in the HLA region contribute to predisposition to typical type 1 diabetes. However, previous studies failed to find an influence of HLA on WS [4,5]. A second distinction is the apparent absence of an autoimmune process in WS-associated diabetes [6]. The lack of islet cell antibodies has been reported for most cases and GAD antibodies were negative in all studied, respectively [2,7].

The diabetes mellitus associated with WS is clearly related to loss of β cells in the pancreas [8,9]. In one series of an autopsy study, loss of β cells or atrophy of the islets was noted in 9 of 11 of WS patients [8]. The exocrine portion of the pancreas was reported to be normal with the exception of focal areas of fibrosis [9]. Immunohistochemical studies of the pancreas reveal normal staining for glucagon, somatostatin, and pancreatic polypeptide but the virtual absence of cells staining for insulin. This indicates a selective β cell loss with preservation of α and δ cells in the islets [9] and allows for the conclusion that diabetes in WS is caused not by a functional defect in the β cells, but by actual β cell depletion.

In our case, HLA typing of DR was DR-4 and DR-8 which is an increased risk for Japanese juvenile-onset type 1 diabetes [10]. In addition, serological examination showed positive GAD and IA-2 antibodies. These results suggest that the diabetes in our patient was caused by an immune-mediated destruction of the insulin-producing β cells of the pancreas in addition to a selective β cell loss not been described previously in this syndrome.

Many mutations, along the entire gene, with homozygous and compound heterozygous mutations, have been described since the identification of *WFS1* as the cause of WS. The patient had a novel homozygous

insertion mutation causing a frameshift at codon 371 leading to premature termination at codon 443, resulting in a complete absence of the carboxy tail of the *WFS1* protein. Although function of *WFS1* gene has not been fully elucidated, a role in membrane trafficking, protein processing, or calcium homeostasis in the endoplasmic reticulum has been postulated [11].

Furthermore, it is speculated that the carboxy tail is interacting with other, unknown proteins [11]. Expression studies of mutant proteins are necessary to determine which parts of the protein are essential for biological function.

In summary, we have reported a case of WS patient carrying a novel mutation in *WFS1* gene with positive GAD and IA-2 antibodies. In the present case, the diabetes might be caused by an immune-mediated destruction and a selective loss of the insulin-producing β cells of the pancreas. In addition, further molecular analysis is necessary to uncover the pathogenesis of this syndrome.

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