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FIGURE LEGENDS

Figure 1. Activation of three subpathways of the UPR in *wfs1*-deficient islets.

A) Activation of the PERK/eIF2α pathway. Islets isolated from wild-type and *wfs1*-deficient mice were subjected to SDS-PAGE and probed with the indicated antibodies: P-PERK, phosphorylated-PERK; P-eIF2α, phosphorylated-eIF2α; T-eIF2α, total eIF2α.

B) Real-time RT-PCR analysis of GRP94, GRP78 and $P58^{IPK}$ gene expressions in wild-type (open columns) and *wfs1*-deficient (closed columns) mice. Relative mRNA levels were obtained after normalization to actin mRNA. $*p < 0.05$. $n = 6$.

C) Expressions of chaperone proteins in *wfs1*-deficient islets. Lysates of isolated islets were probed with the indicated antibodies.

D) Increased XBP1 mRNA splicing in *wfs1*-deficient islets. Amplification of XBP1 mRNA from islet total RNA with specific primers yields spliced (303 bp) and non-spliced (329 bp) XBP1 transcripts.

E) Activation of the IRE1/XBP1 pathway. Lysates of isolated islets were probed with the indicated antibodies.

Western blot data shown are representative of at least three experiments with different sets of samples.

Figure 2. Increased UPR and its reversal by expression of WFS1 or GRP78 in an SV40 transformed *wfs*1-deficient β-cell line (MIN6wfs1$^{-/-}$).

A) Expression of UPR-related proteins in various MIN6 cell lines. MIN6, MIN6wfs1$^{+/-}$-1, MIN6wfs1$^{-/-}$-1 and MIN6wfs1$^{-/-}$-2 cells were lysed and probed with the

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.

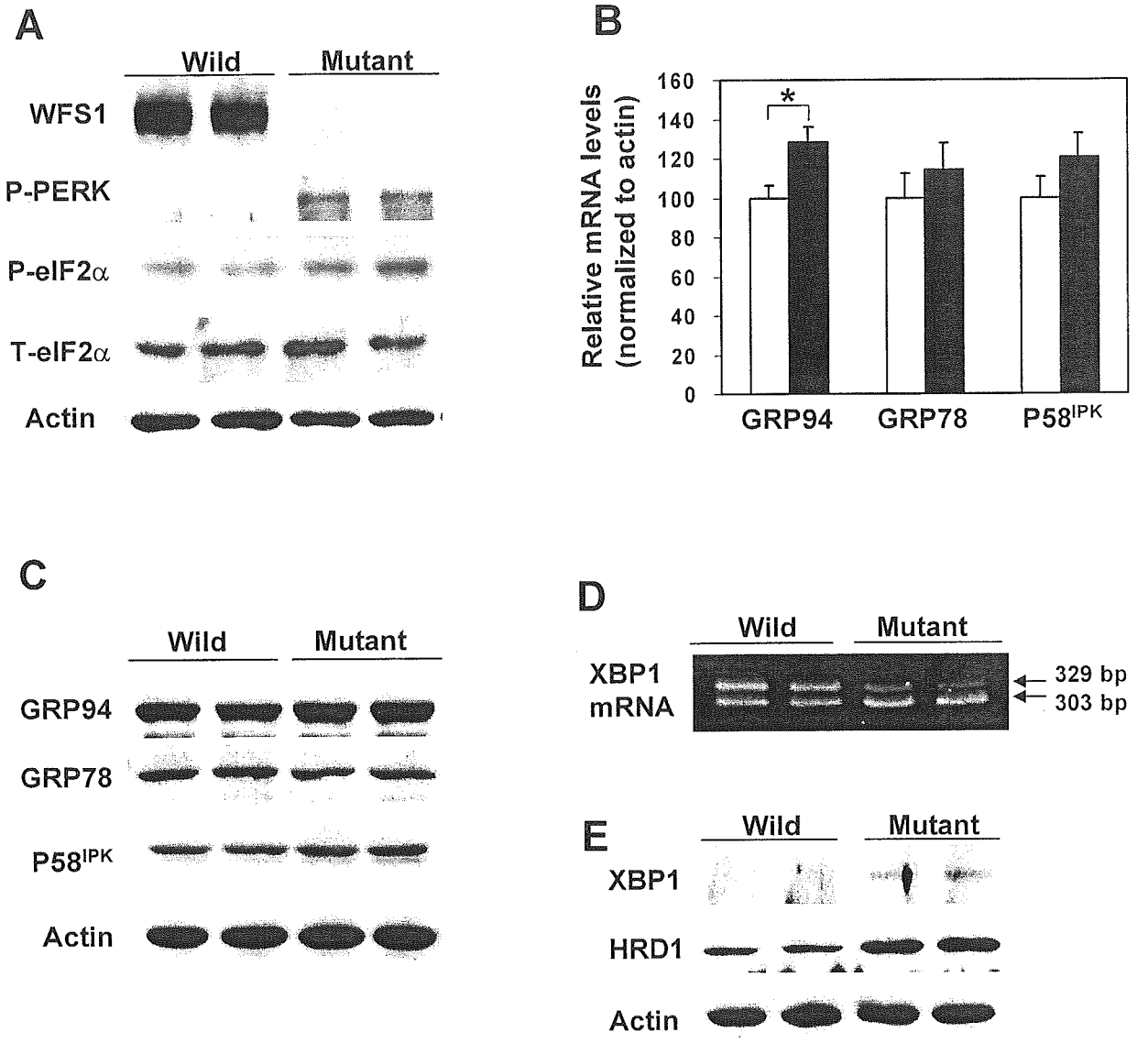
Figure 6. Decrease in MIN6 cell numbers in response to forced $p21^{CIP1}$ expression.

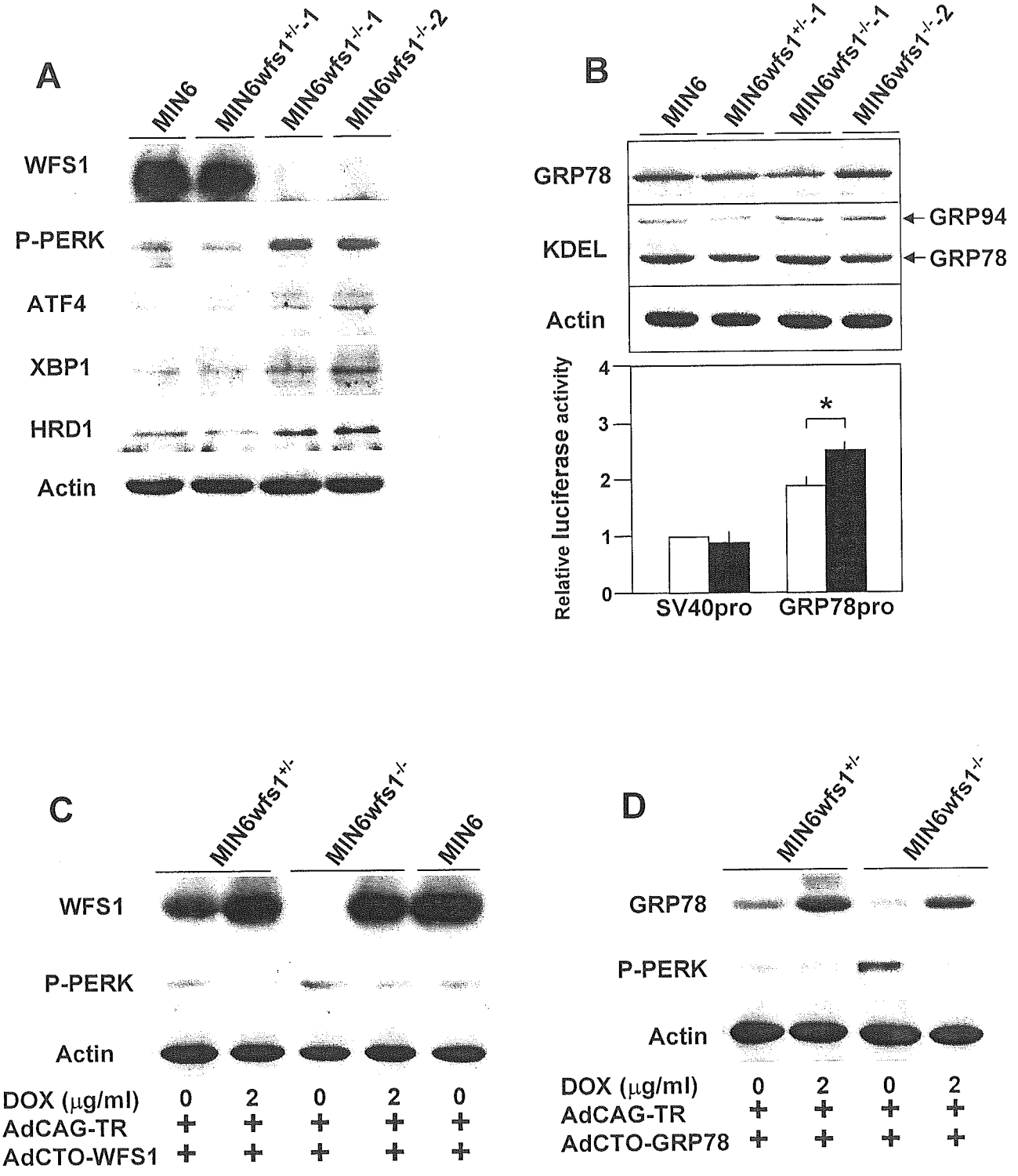
A) Forced expression of $p21^{CIP1}$ in MIN6 cells. Cells were either uninfected or infected with AdCAG-TR (m.o.i. of 30) and AdCTO-$p21^{CIP1}$ (m.o.i. of 100) harboring $p21^{CIP1}$ cDNA. Expression of $p21^{CIP1}$ was induced by 48 hour DOX (2 μg/ml) treatment. MIN6 cell lysates were subjected to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

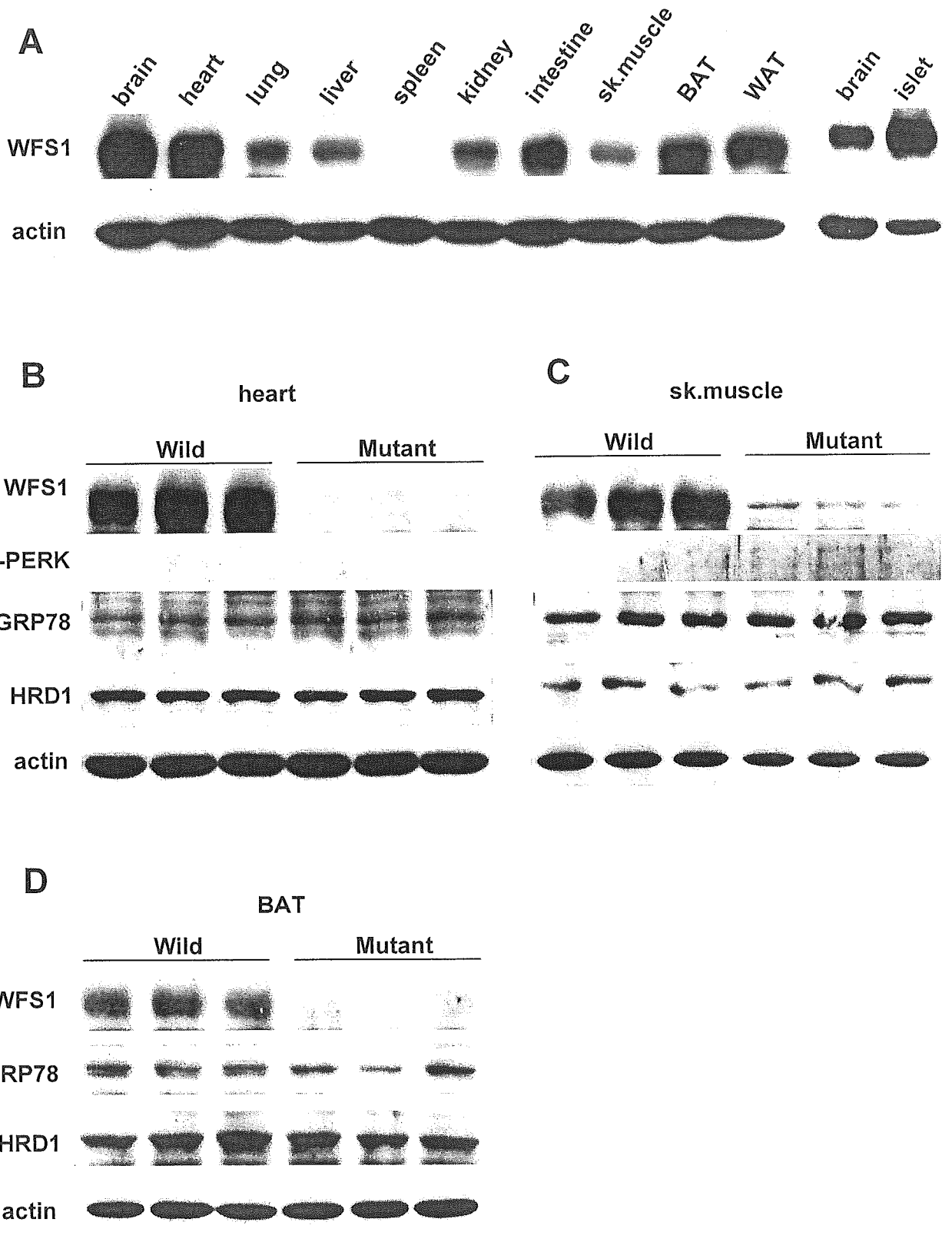
B) Numbers of MIN6 cells overexpressing $p21^{CIP1}$. One day after adenovirus transduction, cells were reseeded (2×10^5 per well) and divided into two groups, and, after two more days, treatment with (closed circles) or without (open circles) DOX (2 μg/ml) was commenced (day 0). Uninfected MIN6 cells (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.

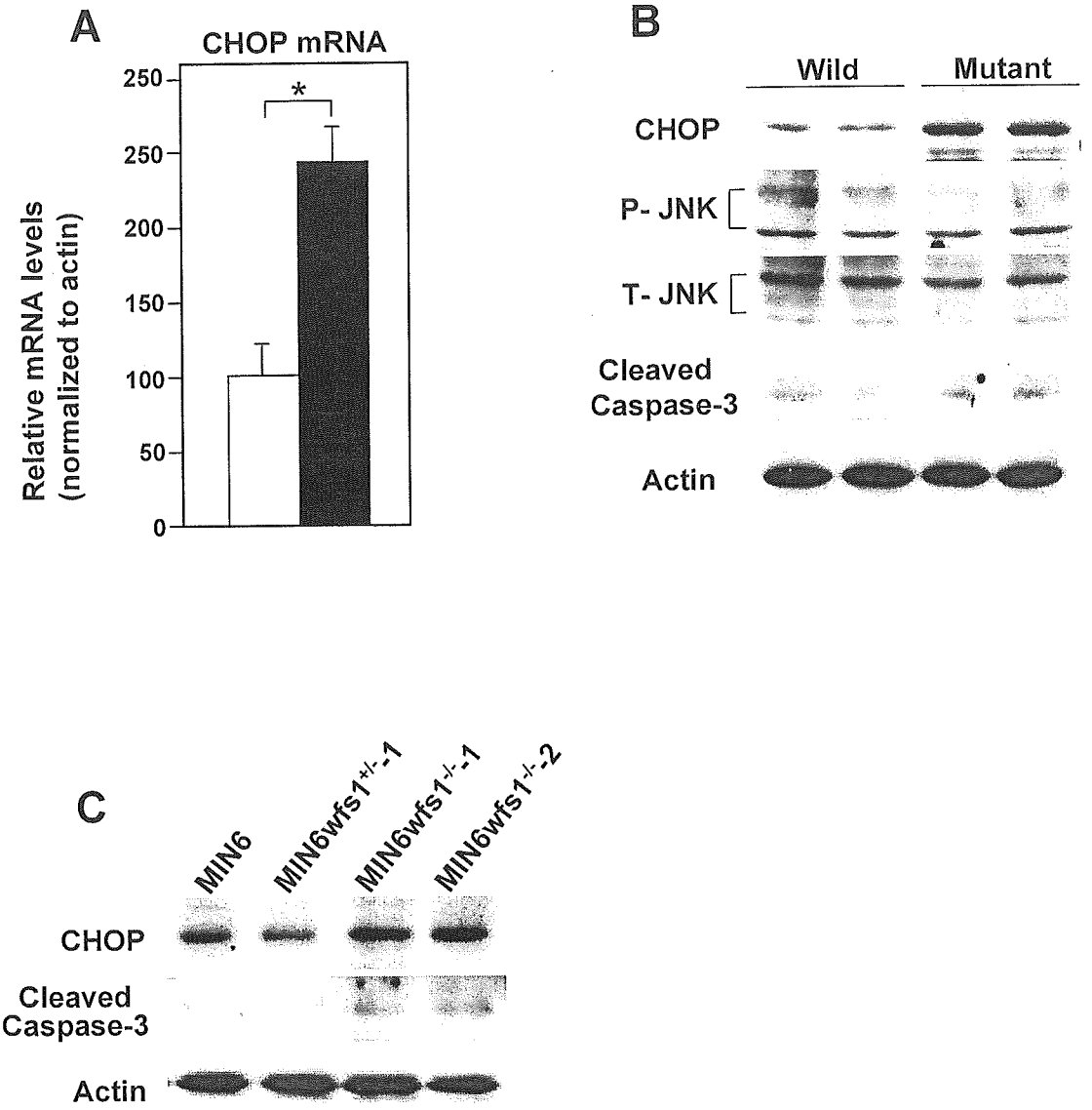
Table 1. Primers used for quantitative real-time RT-PCR

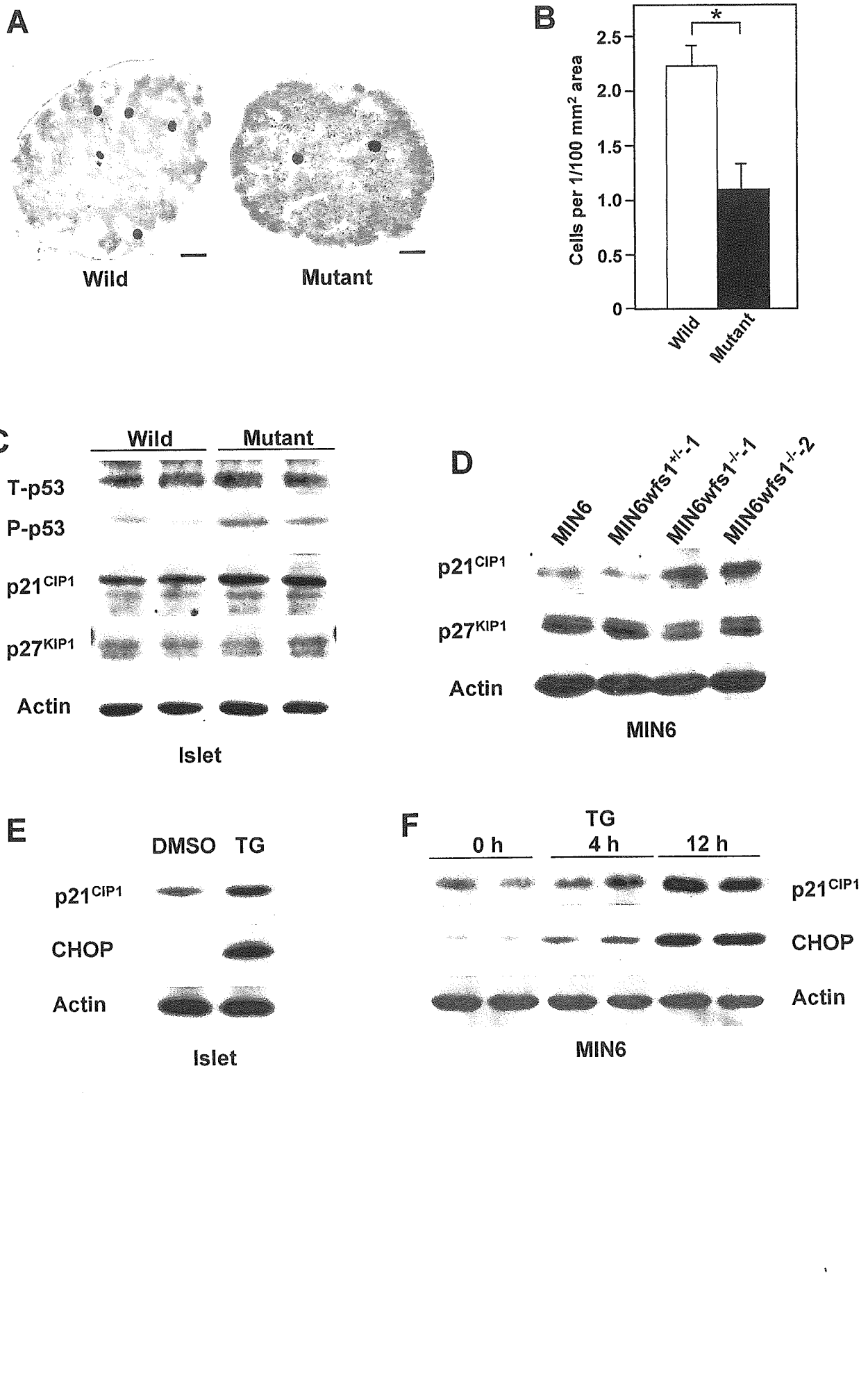
Genes	Forward	Reverse
ATF4	5'-TCCTGAACAGCGAAGTGTTG-3'	5'-ACCCATGAGGTTTCAAGTGC-3'
GRP94	5'-TGATGAAGTCGACGTGGATG-3'	5'-TCCTGTTCACTTCAGCTTGG-3'
GRP78	5'-GACATTTGCCCCAGAAGAAA-3'	5'-CTCATGACATTCAGTCCAGCA-3'
$P58^{IPK}$	5'-CCTTATCGGACAGTCCTTCG-3'	5'-TCAGAGTCCTGATTTCATCTTCA-3'
EDEM	5'-GGAAATTCATCCGAGTTCCA-3'	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

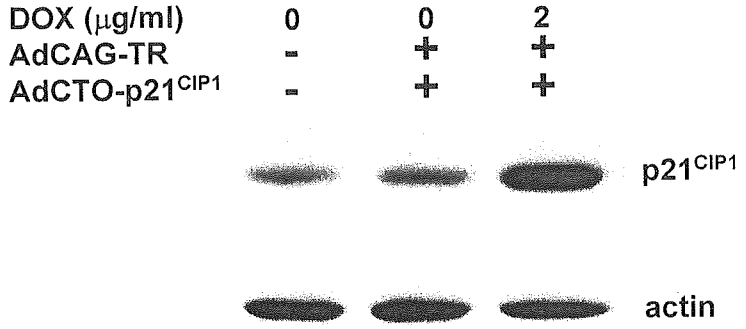

A
MIN6
MIN6wfs1+/- -1
MIN6wfs1-/- -1
MIN6wfs1-/- -2
WFS1
P-PERK
ATF4
XBP1
HRD1
Actin
B
MIN6
MIN6wfs1+/- -1
MIN6wfs1-/- -1
MIN6wfs1-/- -2
GRP78
KDEL
GRP94
GRP78
Actin
Relative luciferase activity
0
1
2
3
4
*
SV40pro
GRP78pro
C
MIN6wfs1+/-
MIN6wfs1-/-
MIN6
WFS1
P-PERK
Actin
DOX (μg/ml) 0 2 0 2 0
AdCAG-TR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

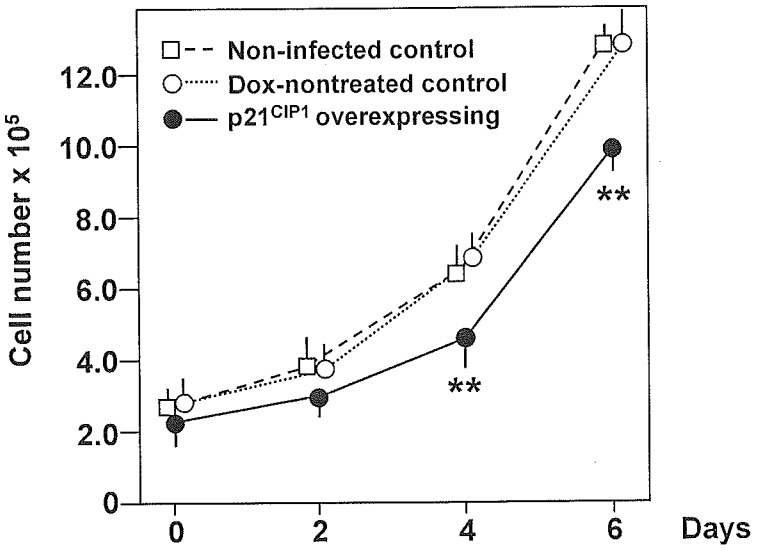

A
CHOP mRNA
Relative mRNA levels (normalized to actin)
250
250
200
150
100
50
0
*
B
Wild
Mutant
CHOP
P- JNK
T- JNK
Cleaved Caspase-3
Actin
C
MIN6
MIN6wfs1+/- -1
MIN6wfs1-/- -1
MIN6wfs1-/- -2
CHOP
Cleaved Caspase-3
Actin


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.

4. ミトコンドリア遺伝子異常による感音難聴

4.1 はじめに

ヒトのミトコンドリアDNAは16, 569bpの環状二本鎖で、22種の転移RNA（tRNA）遺伝子と2種のリボソームRNA（rRNA）遺伝子のほかに13種の酸化的リン酸化酵素複合体のサブユニットがほぼ隙間なくコードされている（図7.4.1）。一つの細胞あたり数千コピーも存在しており、完全な母系遺伝を示す。ミトコンドリア病では変異型DNAと野生型DNAが共存している状態（heteroplasmy）がしばしば見られる。

ミトコンドリア遺伝子の障害により難聴が生じうるが、どの遺伝子の異常でも難聴が生じるわけではない。表7.4.1に難聴を来す代表的なミトコンドリア遺伝子異常とその随伴症状を示す。難聴の三大疾患、MELAS(mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes)、MERRF（myoclonus epilepsy associated with ragged-red fiber)、CPEO(chronic progressive external ophthalmoplegia)では症例の約半数に難聴が生じるとされる[1]。1555位A→G点変異ではアミノ配糖体抗生物質による蝸牛の易受傷性が亢進し、少量のアミノ配糖体抗生物質（AG）投与で難聴を生じるが、この変異家系内にはAG未使用で難聴を生じることもある[2,3]。またこの変異が心筋症やパーキンソン病に関与する可能性も報告されている[4,5]。3243位A→G点変異はMELASのほかに糖尿病・難聴（maternally-inherited diabetes and deafness：MIDD）家系の原因となる[6~8]。7445位点変異は難聴のみ生じると初期に報告されたが、palmoplantar keratodermaを来すことが後に示されている[9]。7472位のheteroplasmic insertion-mutationでは難聴の他に晩発性の神経症状を来すことが報告されている[10,11]。7511位の点変異では難聴以外の症状はまずないとされる[12,13]。

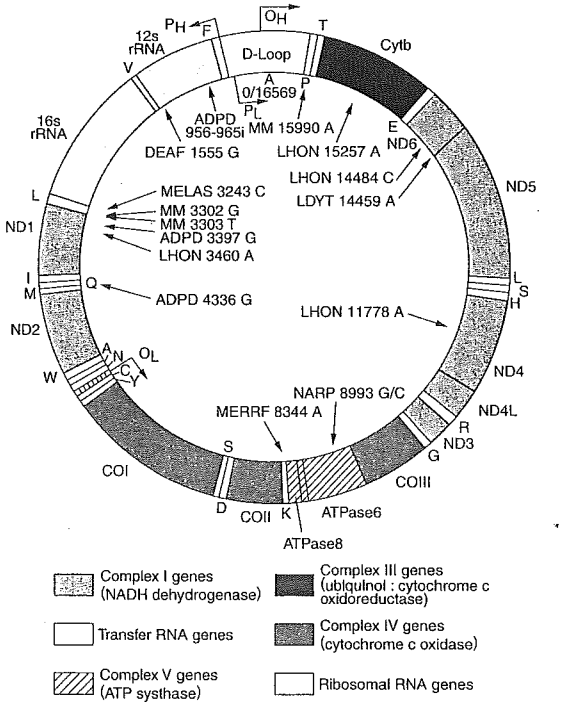


図7.4.1　ミトコンドリアDNAの構造と主要な疾患の遺伝子異常の部位（Wallace DC. Proc Natl Acad Sci USA 91: 8739-8746, 1994より引用）

4.2 難聴発症の機序（仮説）

4.2.1 総論

ミトコンドリア遺伝子異常の障害は赤色ぼろ線維（ragged-red fiber, RRF）を筋病理の特徴として示すもの（MELAS, MERRF, CPEOなど）と蛋白質をコードする遺伝子の変異によって生じ、RRFなどを来さない純粋なencephalopathy（Leigh脳症など）に大きく区分される。難聴は前者で約半数と高率に見られるが、後者ではみられない。酸化的リン酸化は両者ともに障害されるが、ミトコンドリアの蛋白合成は前者でのみ障害されることから、難聴はRRFの発現と類似の機序（蛋白合成障害など）によって生じていることが示唆される。AGによる難聴との関係が主に注目されている1555位A→G点変異症例の骨格筋でも、cytochrome c oxidase（COX）活性低下を伴うmoth-eaten像、封入体を含むミトコンドリア形態異常、少量のRRF（図7.4.2)[3]、NADH-dehydrogenaseとCOXの活性低下[4]、complex I activityの低下[5]などが報告されており、難聴のみしか呈さない7511点変異の骨格筋でもCOX活

表7.4.1　難聴を示す代表的なミトコンドリア遺伝子異常

ミトコンドリア遺伝子異常(主)	随伴する臨床症状または診断
delT961Cn	AG感受性亢進
A1555G	AG感受性亢進、心筋症、パーキンソン病
A3243G	MELAS、MIDD（糖尿病）
A7445G	palmoplantar keratoderma
7472insC	晩発性神経症状
T7511C	−
A8344G、T8356C	MERRF
Heteroplasmic large deletion	CPEO(Kearns-Sayre syndrome)

性低下が見られている[12,13]。

4.2.2　1555点変異でのAGによる難聴

AGの作用機序にはリボソームにおける蛋白合成阻害と細胞膜障害があるが、前者が主である。AGはリボソームに不可逆的に結合してmRNAの情報を読み誤まらせ、蛋白合成を阻害する。ヒトのミトコンドリアDNA1555位近傍は細菌から哺乳類まで保存された構造で、AGのrRNAに対する結合部位の一つであり、この部位の突然変異が細菌におけるAG耐性を来すことが知られる[14]。ヒトでは1555位A→G点変異により12SrRNAの構造が変わってAGがつきやすくなることが示唆されている[15]。我々が1555位A→Gサイブリド細胞と変異を持たないサイブリド細胞をAG存在下で培養したところ、1555位A→G細胞ではより低AG濃度での生育阻害、呼吸鎖活性の顕著な低下、ミトコンドリア翻訳活性の低下を認め、AGはミトコンドリアの翻訳系に直接作用し、1555位A→G変異はAG感受性を高めることを明らかにした[16]。また[^{35}S]メチオニンを用いたパルスラベルによるミトコンドリアの翻訳産物解析では1555位A→G細胞においてAG存在下で異常な翻訳産物が産生すること、および大腸菌のリボソーム（1555位変異を有するモデルリボソーム）ではUAGコドンを読み飛ばしやすいことが判明しており、1555A→G変異の存在下ではAGがミトコンドリアの翻訳精度の低下を引き起こし、異常な翻訳産物を生産させることが示唆されている。

硫酸ストレプトマイシン（SM）は通常前庭障害を優位に生じるが、1555位A→G点変異症例ではSMの少量投与により前庭・半規管機能は保たれたまま、まず感音難聴が出現する[2,3]。このことはAG投与で内耳障害が生じる機序が1555位A→G点変異症例と正常例で異なることを意味する。蝸牛の代謝活性は前庭の数～10数倍であり、蝸牛の血管条や感覚上皮ではK^+、Na^+、Ca^{2+}などのイオン輸送が盛んで、これに要するATPが常にミトコンドリアで産生されている。このためミトコンドリア機能の障害は蝸牛により影響を及ぼしやすい。1555A→G点変異ではAGがミトコンドリア機能障害を生じるため、前庭障害をまだ来さない少量の投与量においても蝸牛障害を生じるものと考えられる。

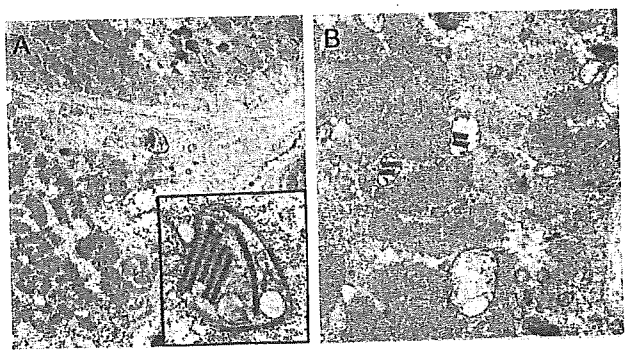


図7.4.2　A1555G点変異症例に見られた筋病変（透過電顕像）[3]
Moth-eaten fiberに見られた封入体を含む拡大したミトコンドリア（A: × 8000、inset: × 40000、B: × 23000）

4.3　病態

ミトコンドリア遺伝子異常により生じるため、難聴は母系遺伝（女性からは次の世代に伝わるが、男性からは伝わらない）を示す。稀ではあるが孤発例も存在する。

難聴は主として蝸牛障害により生じ、後迷路障害は稀である[2,3,6]。多くは思春期以降に両側に出現し、徐々に進行する。時に急速に悪化することもある。3243位A→G点変異では水平型か高音漸傾型が多く（図7.4.3）[6]、1555位A→G点変異では高音急墜型が多い[2,3]。1555位A→G点変異にAGを投与した場合は少量投与で難聴が出現し、投与終了後も難聴が進行する[2,3]。なおAGを含む点耳薬を鼓膜穿孔例に投与した場合も少量投与で難聴が出現し、投与終了後に難聴が進行することがあるが、これは1555A→G点変異とは関係なく、蝸牛窓経由でAGが蝸牛内に蓄積することが原因と思われる[17]。一般にミトコンドリア病ではheteroplasmy（変異DNAの割合）と症状発現に相関のあることが知られているが、難聴との関係を調べたものは少ない。我々が3243位A→G点変異症例でheteroplasmyと難聴の関係を調べたところでは、変異DNAの比率が高いほど難聴の発症年齢は若かったが、難聴の進行速度との間に相関は見られなかった[6]。平衡機能は保たれていることが多く、障害される場合も徐々に両側性に生じるためか症状を訴えることは少ない。

4.4　側頭骨病理

ミトコンドリア遺伝子異常の確認された側頭骨病理は少ない。我々は56歳で死亡したMIDDの側頭骨から3243位A→G点変異の同定に初めて成功した。この症例は49歳時には平均右53dB、左43dBの水平型感音難聴を呈していたが、55歳時には聾になっている。側頭骨病理では蝸牛全体に血管条と外有毛細胞の変性が著しく、ラセン神経節も基底回転で特に消失していた（図7.4.4）が、前庭の変性は軽度であった[8]。2003年に報告されたMELASの2症例の側頭骨病理では、血管条とラセン神経節の高度変性が特徴であり、het-

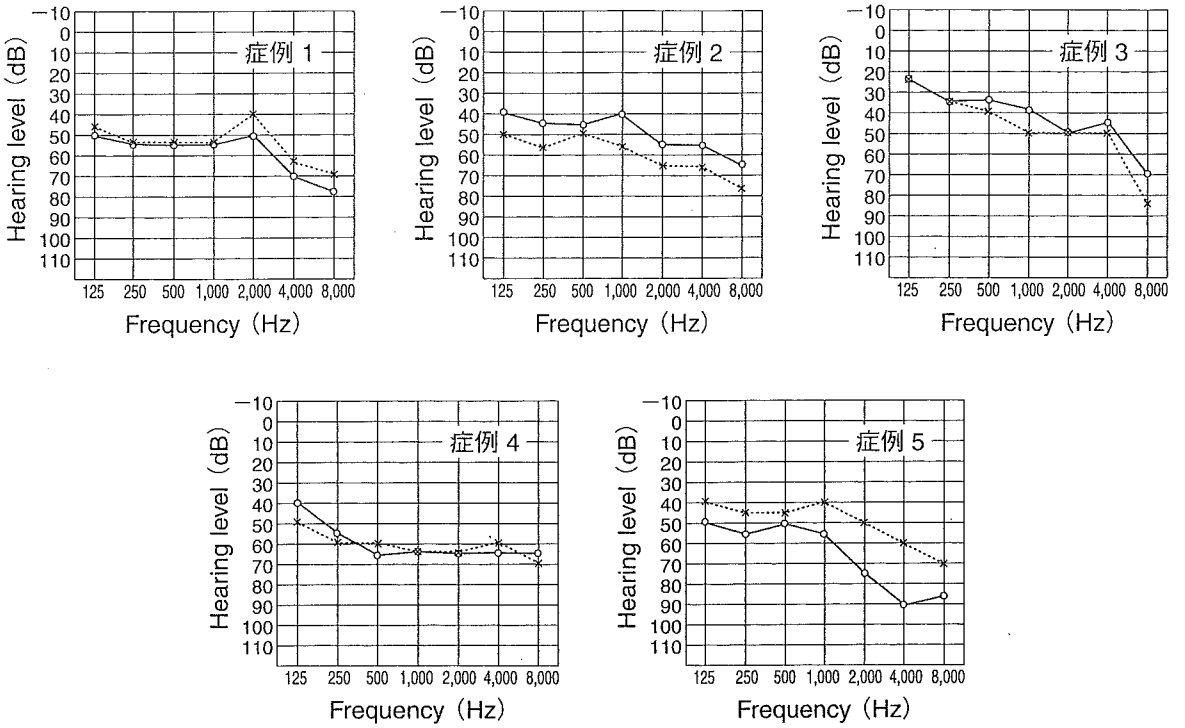


図 7.4.3　3243A → G 点変異症例の聴力像[6]
症例 1 から順に 33 歳女性、38 歳女性、42 歳女性、54 歳女性、61 歳男性であり、難聴の発症はそれぞれ 29 歳、26 歳、33 歳、39 歳、55 歳、heteroplasmy はそれぞれ 29%、30%、26%、7%、8%である。

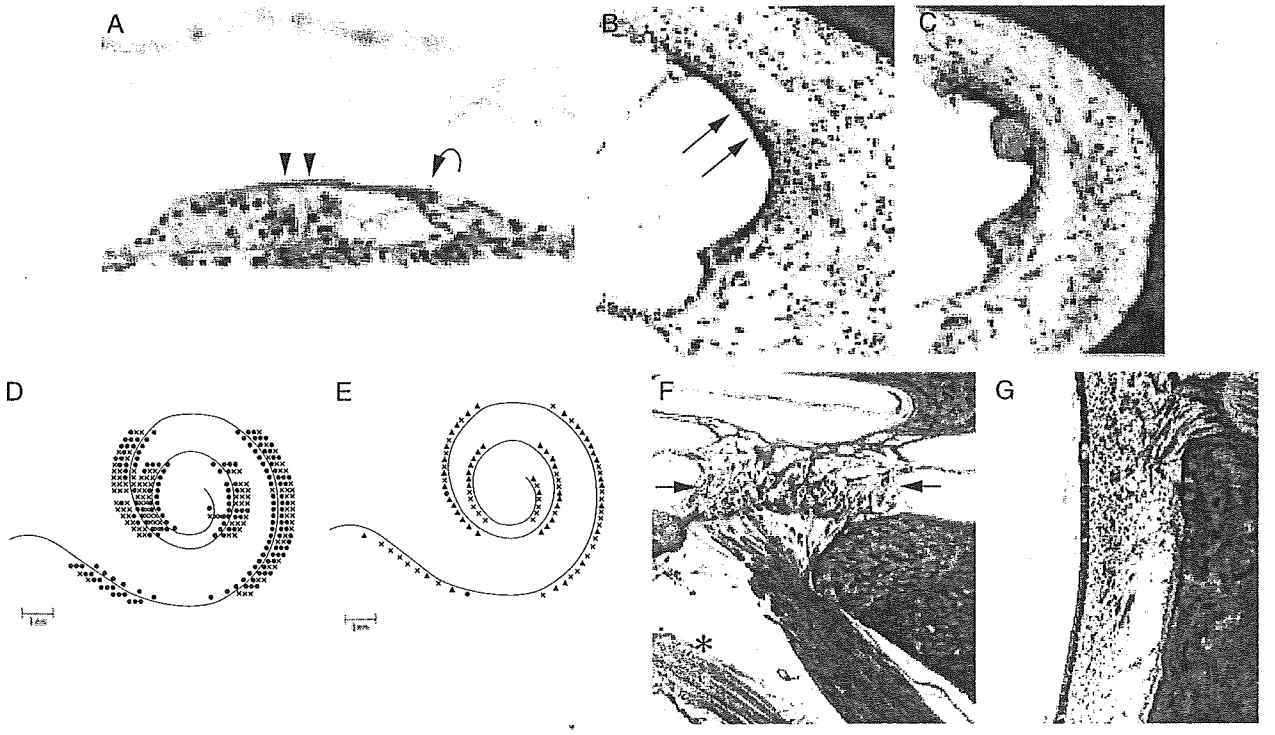


図 7.4.4　糖尿病・難聴の母系遺伝（MIDD、A3243G 点変異）症例の側頭骨病理[8]
A：内有毛細胞（矢印）は保たれるが外有毛細胞（矢頭）は消失している。
B：血管条の萎縮。
C：血管条の嚢胞状変性。
D：蝸牛全体の内外有毛細胞の状態。主に外有毛細胞の障害が見られる（●：存在、×：消失）。
E：蝸牛全体で血管条の変性が見られる（●：変性軽度、▲：変性中等度、×：変性高度）。
F：基底回転でのらせん神経節の変性。
G：球形嚢の感覚上皮はほぼ保たれている。