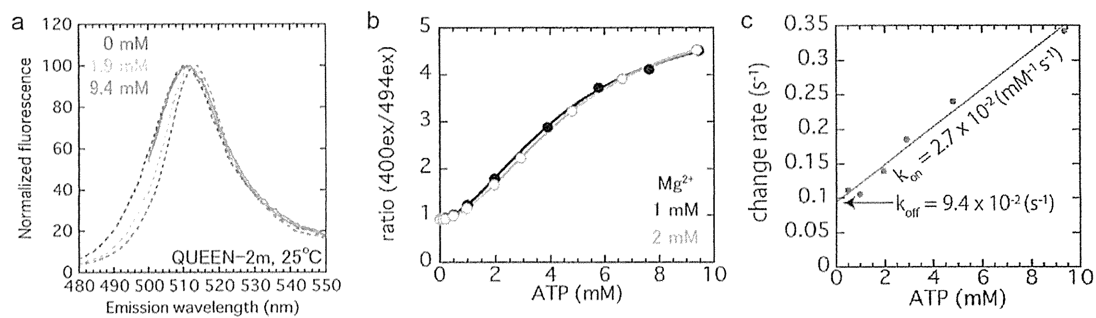

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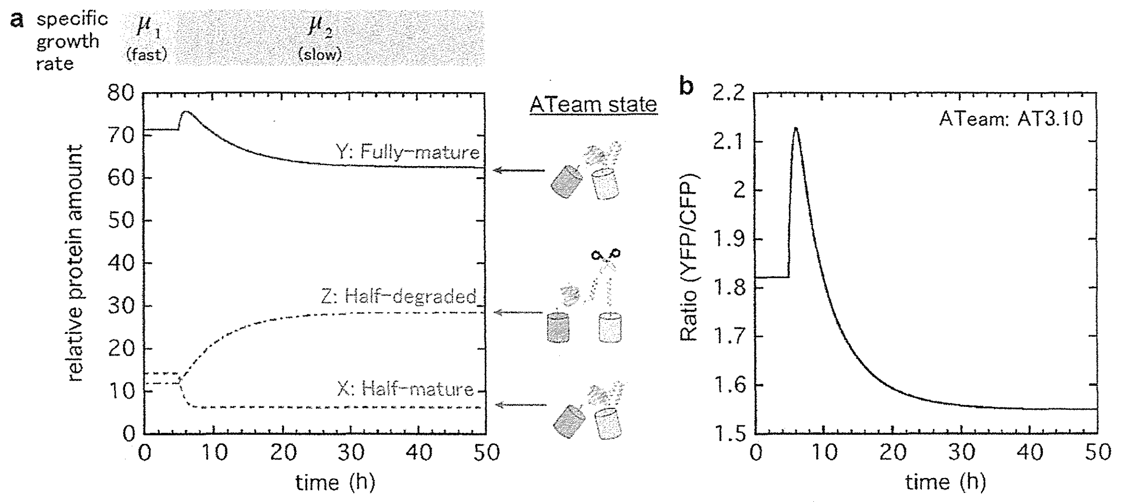

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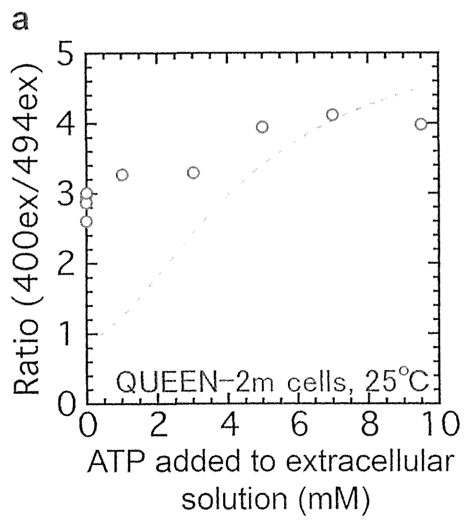

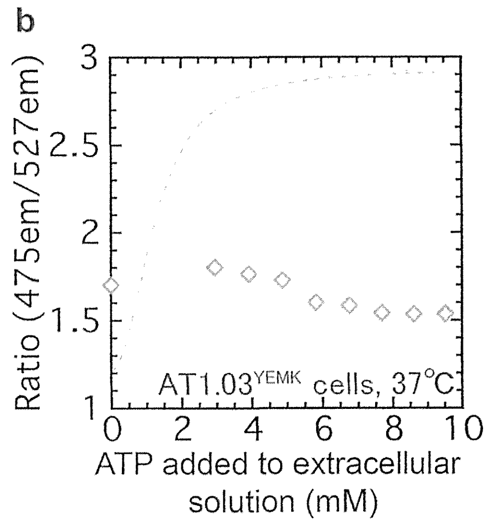

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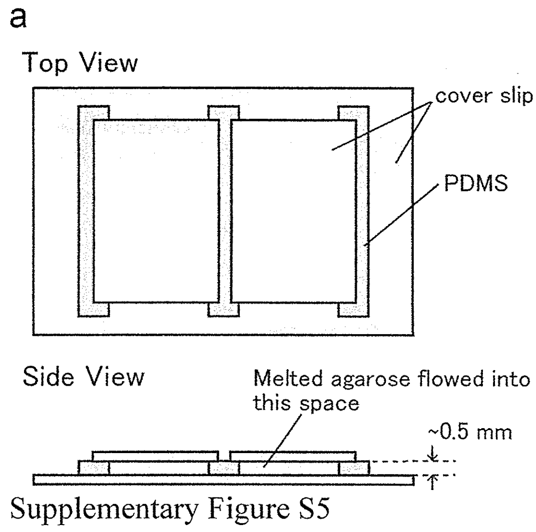

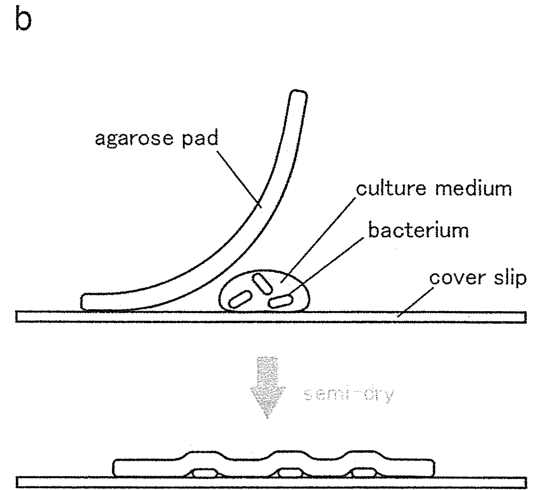

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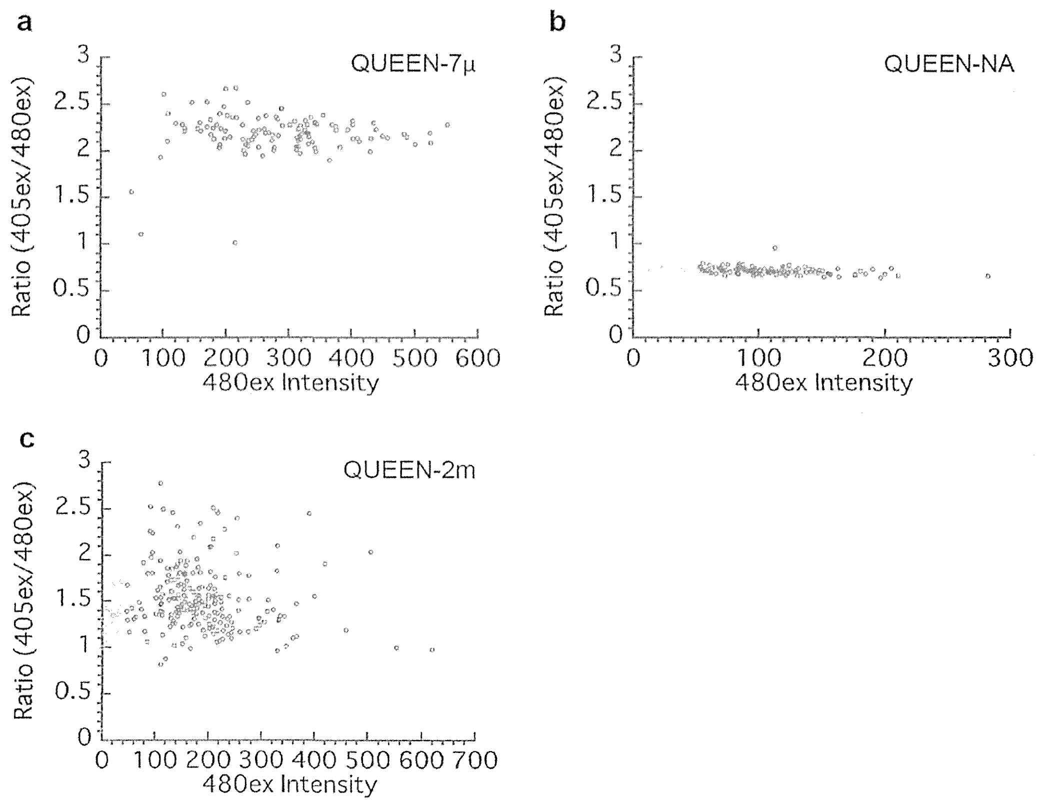

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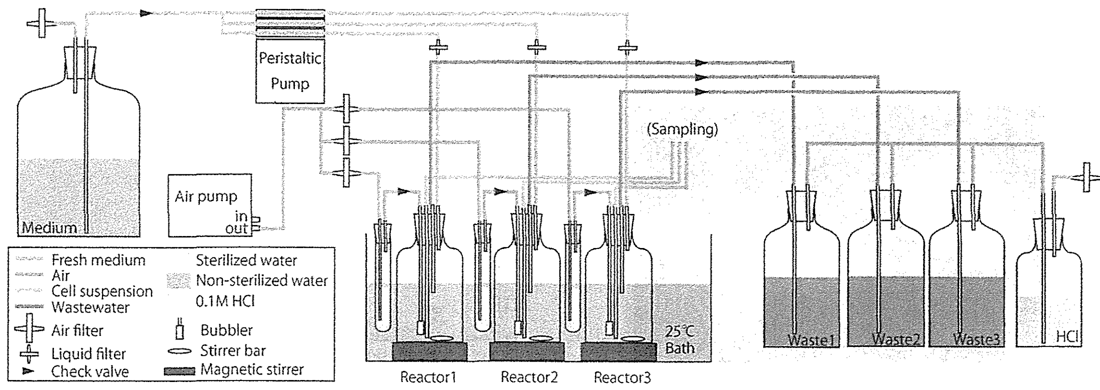

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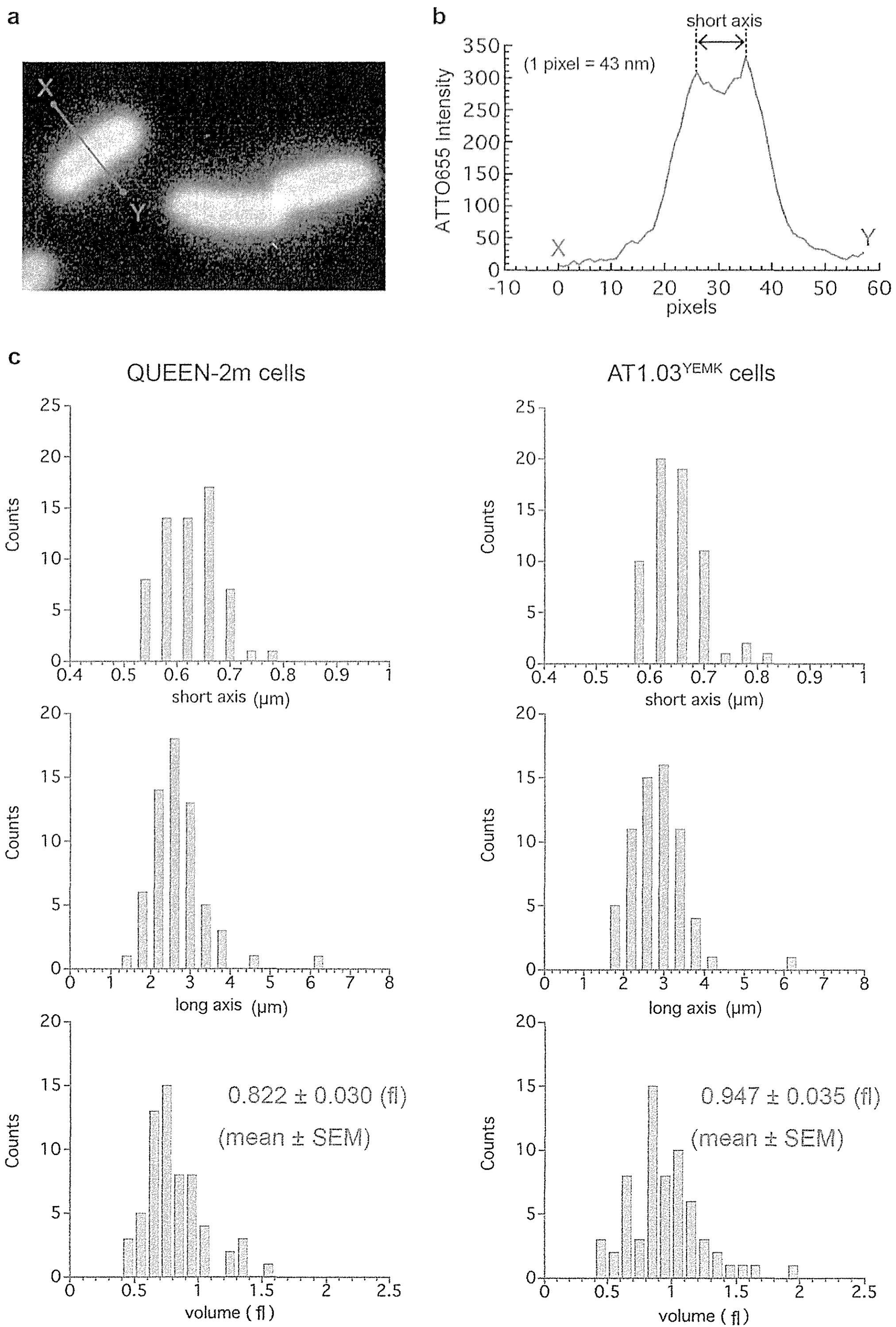

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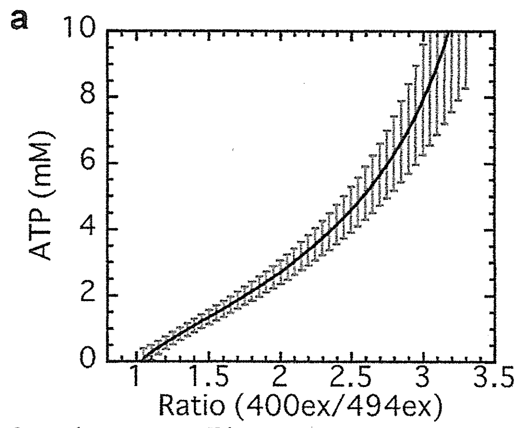

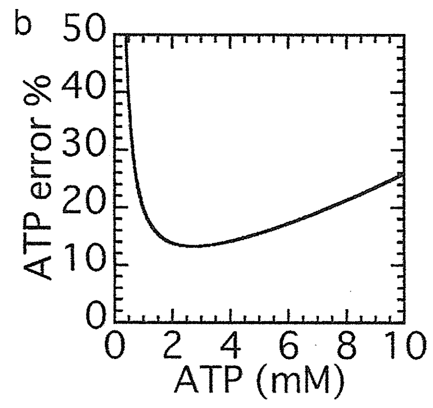

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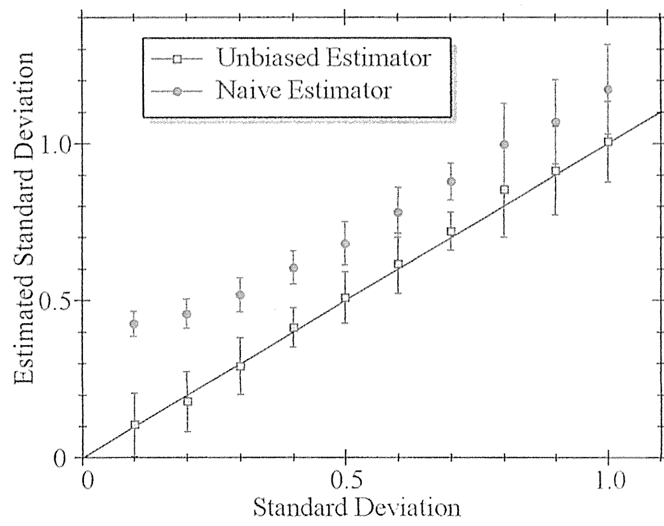

Supplementary Figure S10

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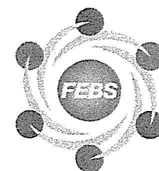

VCP/Cdc48 rescues the growth defect of a *GPI10* mutant in yeast

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ABSTRACT

We identified a yeast mutant with temperature-sensitive growth defects that were rescued by VCP expression. The mutation occurred in *GPI10*, which encodes a mannosyl transferase for glycosylphosphatidylinositol anchor formation in the endoplasmic reticulum, and caused a Gly469Glu substitution in Gpi10. The mutant exhibited increased unfolded protein response, which was partially rescued by VCP or Cdc48, and showed sensitivity against cell-wall stressors, which were not rescued by VCP. These results suggest a potential link between VCP/Cdc48 and Gpi10 functions in the control of cell growth.

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1. Introduction

VCP/p97, which is Cdc48 in yeast, is an abundant, conserved, and essential hexameric ring-shaped AAA$^+$ ATPase [1,2]. The activities of VCP are mainly associated with ubiquitin-dependent processes, including proteasome-mediated protein degradation, membrane fusion, endosomal protein trafficking, autophagy, and genomic DNA surveillance. VCP functions in different sites within a cell, including the cytosol, the nucleus, and the cytoplasmic sides of organelles, such as the endoplasmic reticulum (ER), mitochondria, and peroxisome. These diverse activities are thought to be derived from the ability of VCP to bind >30 cofactors, and these cofactors apparently specify the particular functions of VCP. Not only VCP but also many of these VCP cofactors have ubiquitin-binding motifs, which further support its link to ubiquitin-related activities. In addition to its normal cellular activities, many single missense mutations of VCP cause two autosomal dominantly inherited diseases with neurodegeneration, namely, inclusion body myopathy associated with Paget's disease of the bone and frontotemporal dementia (IBMPFD) [3,4] and a rare familial form of amyotrophic lateral sclerosis (ALS) [5].

Mammalian VCP and yeast Cdc48 have been reported to function in many similar pathways. To find novel VCP/Cdc48 functions, we screened for mutants that had temperature sensitivities and growth defects that were suppressed by VCP expression, and we identified a mutation in *GPI10*, which encodes a mannosyl transferase in the ER.

2. Materials and methods

2.1. Media

The yeast was grown in YPAD medium (1% yeast extract, 2% bactopeptone, 2% glucose, and 0.004% adenine) in synthetic complete medium (SD; 0.67% yeast nitrogen base and 2% glucose supplemented with amino acids) or synthetic casamino medium (SC; 0.67% yeast nitrogen base, 2% glucose, 0.5% casamino acids, and, if necessary, tryptophan, uracil, or adenine). For SGal medium, the glucose in SD was replaced with galactose. To eliminate the *URA3* plasmid, 5-fluoroorotic acid was added to the media at a concentration of 0.5 mg/ml.

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2.2. Yeast strains

The wild-type yeast strain used in this study was W303 (MATa *ade2-1 can1-100 his3-12,16 leu2-3,112 trp1-1 ura3-1*). During the screening, W303 with a VCP expression plasmid was mutagenized with ethyl methanesulfonate (EMS; Sigma–Aldrich Co. LLC, St. Louis, MO, USA) and plated on glucose at 25 °C. The viability of EMS-treated cells was 49% compared to that of non-treated cells. Among approximately 40 000 EMS-treated cells, colonies that grew on galactose but not glucose at 37 °C were selected. We obtained one strain from the screening, and the strain was crossed with W303 three times to generate the 1–30 strain (*gpi10-2*) to eliminate extraneous mutations. During the screening, we also tried to generate strains that grew on glucose but not on galactose at 37 °C, but we were unable to generate such strains.

2.3. Plasmid construction

A list of the plasmids used in this study is provided in Supplementary Table 1. A plasmid expressing VCP under the *GAL1* promoter (M123) was created as follows: the EcoRI-BamHI fragment of pRS316 was ligated to the EcoRI-BamHI fragment of the *GAL1* promoter to create V039. The pCMX-VCP-GFP plasmid was cut with Asp718 and BamHI and blunted and ligated with a BamHI fragment of V039, which had been blunted. The plasmids expressing VCP or Cdc48 under the *GPD* promoter have been described previously [6]. The pRS316-GPI10(WT) and pRS316-UBI4(WT) plasmids were constructed as described below. The DNA of *GPI10* and *UBI4* were amplified by polymerase chain reaction (PCR) from wild-type genomic DNA and then cloned into pRS316. pRS316-GPI10(*gpi10-2*) was constructed by cloning the amplified *gpi10-2* from the genomic DNA of 1–30 into pRS316. The pR12-28 and pR12-19 plasmids were obtained from the YEP13-based yeast genomic library, which was a gift from Dr. K. Matsumoto.

The pCZY1 plasmid, which contained a *lacZ* reporter gene that was driven by the *CYC1* core promoter and that was fused with the unfolded protein response (UPR) element (UPRE) to monitor UPR activity [7], was a gift from Dr. Y. Kimata.

2.4. Growth assay of yeast

For the spot assay, the cell densities were adjusted to OD600 = 1.0 and diluted serially by 10-fold dilutions. Each diluted sample was spotted on the indicated plates and incubated for 2 or 3 days at the indicated temperatures.

2.5. β-Galactosidase assay for UPR response

The β-galactosidase activity for the UPR response was determined according to the protocol of Kimata et al. [8].

3. Results

3.1. Screening of the temperature-sensitive mutants with growth defects that were rescued by VCP overexpression

To find novel VCP/Cdc48 functions and eventually find proteins that are functionally related to VCP/Cdc48, we generated yeast temperature-sensitive (ts) mutants that had growth defects that were suppressed by the expression of human/mouse VCP (Fig. 1A). Briefly, wild-type cells with a plasmid expressing VCP under the control of the *GAL1* promoter, which functions in galactose media but not in glucose media, was treated with EMS and the colonies were replicated. Each replica was plated onto glucose or galactose media. Colonies that grew in galactose but not in glucose

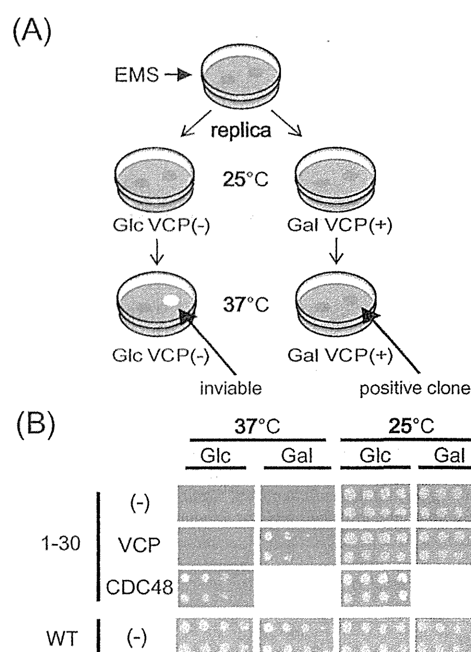

Fig. 1. Isolation of the temperature-sensitive (ts) mutant with ts growth defects that were rescued by VCP and Cdc48. (A) Screening strategy. Wild-type cells with a plasmid expressing VCP under a galactose-inducible promoter (GAL1p) were mutagenized with ethyl methanesulfonate. Ts mutants were screened on glucose and for suppressing ts on galactose at 37 °C. (B) Isolation of the ts strain 1–30. Cells with or without exogenously expressed VCP and Cdc48 were diluted, spotted on synthetic casamino medium (SC)-ura or SGal-ura plates, and grown at the indicated temperatures for 3 days.

media at 37 °C were selected. From about 40 000 EMS-treated cells, we obtained one strain (referred to as 1–30), which was able to grow only in the presence of VCP expression at 37 °C (Fig. 1B). In addition to VCP, the overexpression of yeast Cdc48 also rescued the growth defect of strain 1–30 at 37 °C (Fig. 1B), which suggested that some conserved function(s) of the VCP/Cdc48 families were responsible for the suppression.

To identify the mutated gene in the 1–30 strain, we screened yeast genomic multicopy libraries for plasmids that would complement its ts phenotype. One plasmid (pR12-28) allowed the 1–30 strain to grow at 37 °C (data not shown). A deletion analysis of pR12-28 identified the suppression activity in a region containing the entire *GPI10* open reading frame and its flanking regions (Fig. 2A). Another plasmid pR12-19 rescued the ts phenotype of 1–30 but not as efficiently as pR12-28 did (data not shown). A deletion analysis of pR12-19 identified a region containing *UBI4*, which is a polyubiquitin gene, and its flanking region for the partial ts-suppressing activity. Moreover, we found that the expression of *UBI4* by a multicopy plasmid [pRS426-UBI4(WT)] but not by a single-copy plasmid [pRS316-UBI4(WT)] was required for the efficient suppression of the temperature sensitivity of the 1–30 mutant (Fig. 2B).

3.2. Identification of the mutation in GPI10 in the 1–30 strain

The efficient suppressive activity that occurred by the introduction of *GPI10* in a single-copy plasmid suggested that the mutated gene in strain 1–30 was *GPI10*. Therefore, we amplified *GPI10* and its flanking region by polymerase chain reaction (PCR) from 1–30 genomic DNA and then tested for ts suppression in a single-copy plasmid. Indeed, it failed to rescue the growth defect of strain 1–30 at 37 °C (Fig. 2A, bottom). In contrast, *UBI4* and its flanking

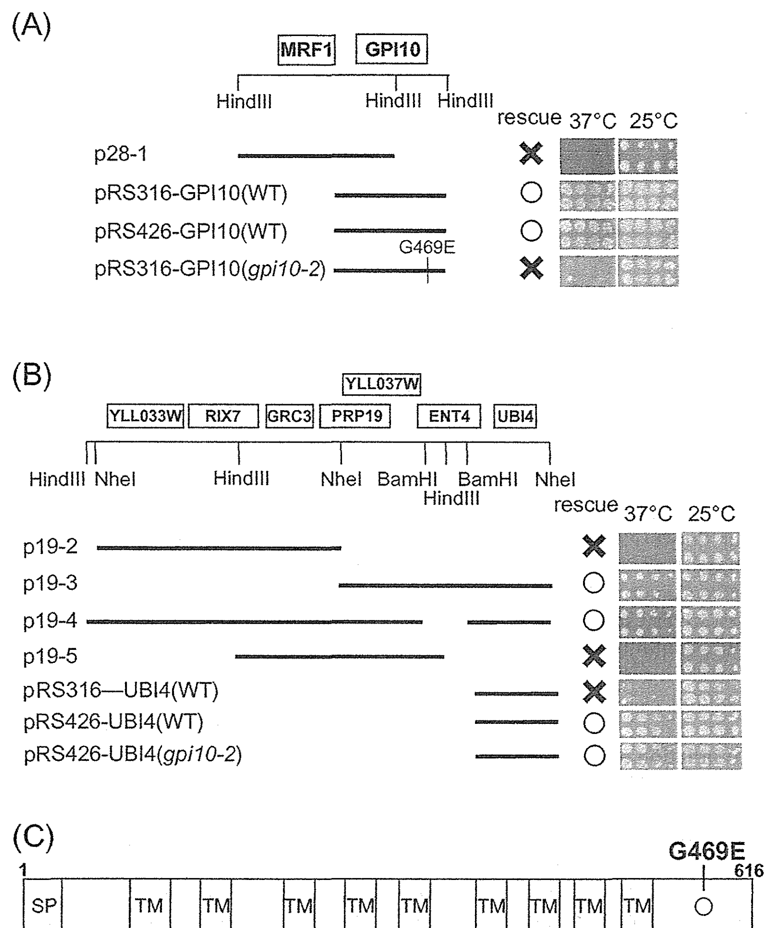

Fig. 2. Identification of the mutation in *GPI10* in strain 1–30. (A) Mapping of the suppressing gene for the 1–30 ts mutant to identify *GPI10*. Plasmids were checked for their ability to suppress the ts growth of 1–30. pRS316-GPI10(WT) and pRS426-GPI10(WT) contained *GPI10* DNA fragments that were obtained by polymerase chain reaction (PCR) as wild-type genomic DNA, and pRS316-GPI10 (*gpi10-2*) contained a PCR-amplified *GPI10* DNA fragment from 1–30 cells. Cells with a plasmid were diluted, spotted on YPD plates, and grown at the indicated temperatures for 3 days. Two independent transformed cells are shown in two columns. (B) Mapping of the suppressing gene to identify *UBI4*. Plasmids pRS316-UBI4(WT) and pRS426-UBI4(WT) contained *UBI4* DNA that was obtained by PCR with wild-type genomic DNA, and pRS426-UBI4 (*gpi10-2*) contained PCR-amplified *UBI4* DNA fragment from 1–30 cells. (C) Localization of the G469E point mutation in the Gpi10 protein. SP indicates a predicted signal peptide, and TM indicates a predicted transmembrane region.

region, which were obtained from 1–30 genomic DNA, rescued the growth defect of strain 1–30 at 37 °C in a multi-copy plasmid [Fig. 2B, pRS426-UBI4(*gpi10-2*)]. These results indicated that strain 1–30 contained the responsible mutation in *GPI10* but not in *UBI4*.

Glycosylphosphatidylinositol (GPI) anchor proteins are membrane integral proteins that function at the cell surface [9,10]. GPI anchors are glycolipids that consist of phosphatidylinositol, glucosamine, mannose, and ethanolaminephosphate, and they are made through many successive enzymatic steps in the ER. After the final step, the resultant GPI-anchor is added to substrate proteins to make GPI-anchor proteins. During the process of GPI-anchor synthesis in yeast, Gpi10, which is a mannosyl transferase, transfers the third mannose to a GPI intermediate containing already two mannoses linked to glucosamine-phosphatidyl inositol, which is an intermediate of the GPI anchor [9]. *GPI10* is essential and encodes a protein with nine transmembrane domains in the ER membrane with a C-terminus region toward the cytosol [11].

By sequencing the *GPI10* gene from the 1–30 mutant, we identified a single nucleotide change from the 1406th guanine to adenine in the *GPI10* gene. This mutation changed the 469th amino acid in Gpi10 from Gly to Glu at and was predicted to be located in the C-terminal cytoplasmic tail of Gpi10 (Fig. 2C) [12]. Introducing the mutation into the *GPI10* gene of wild-type cells conferred a ts phenotype (data not shown). Therefore, we concluded that the mutated gene in strain 1–30 was *GPI10*. The mutated allele in *GPI10* is referred to as *gpi10-2* hereafter because a mutant for *GPI10* (*gpi10-1*) has been previously reported [13].

3.3. Sensitivities of cell-wall stressors in gpi10-2 mutant

Because defects in GPI-anchor proteins lead to a loss of integrity of the cell wall [14,15], we examined the effects of cell-wall stressors on the growth of the *gpi10-2* mutant. As expected, the *gpi10-2* mutant showed enhanced sensitivities to the two cell-wall stressors that were tested, sodium dodecyl sulfate and caffeine,

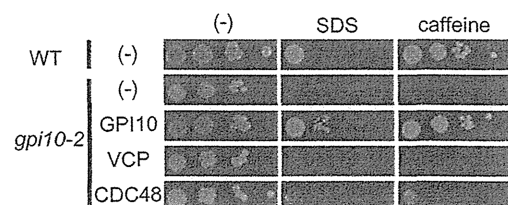

Fig. 3. Sensitivities against cell-wall stressors in the *gpi10-2* mutant. The effect of cell-wall stressors. Wild-type and *gpi10-2* cells with or without exogenously expressed VCP or Cdc48 were diluted, spotted on SD-uracil(−) and SD uracil(−) containing 0.006% SDS or 3 mM caffeine, and incubated at 33 °C for 2 days.

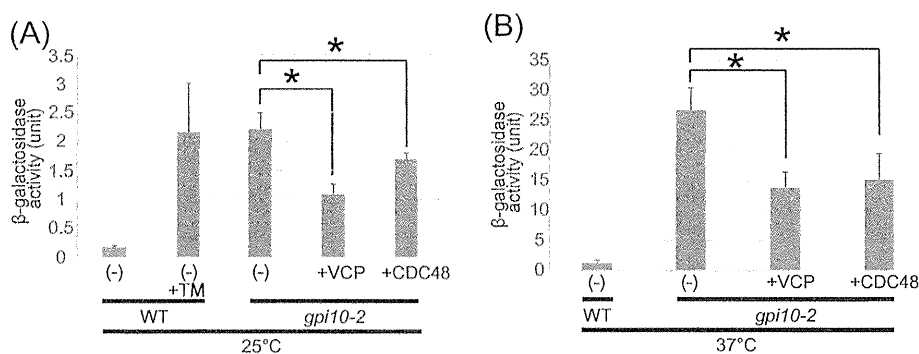

Fig. 4. Increased unfolded protein response (UPR) in the *gpi10-2* mutant. (A) β-gal activities at 25 °C. Cells with or without exogenously expressed VCP or Cdc48 were measured for β-galactosidase activity. Tunicamycin (TM) was added at 2 μg/mL for 2 h. The data are presented as the mean ± standard error of the mean (S.E.) values of four independent experiments. *$P < 0.05$. (B) The β-gal activities after 2 h at 37 °C.

compared to those of wild-type cells, which suggested that GPI anchoring was defective in the *gpi10-2* mutant (Fig. 3).

We examined whether VCP or Cdc48 expression rescued the cell wall-related sensitivities of *gpi10-2*. Unexpectedly, we did not see the recovery of growth defects against the cell-wall stressors by VCP overexpression and saw a very small, if any, rescue by Cdc48 overexpression in the *gpi10-2* mutant (Fig. 3).

3.4. Increased UPR in the gpi10-2 mutant

The UPR is induced by the accumulation of unfolded proteins in the ER [16]. Because the induction of the UPR was reported in deletion mutants of *BST1* and *LAS21*, which are both involved in GPI-anchor production [17], we tested whether the *gpi10-2* mutation would cause the UPR. In the UPR, Ire1, which is a transmembrane protein with endoribonuclease activity, is one of the core mediators of the intracellular UPR signal. Ire1 functions in the splicing of *HAC1* mRNA to produce the translatable mRNA for Hac1, which is a transcription factor that induces ER chaperones. Hac1 directly binds to the UPRE for the expression of ER chaperones. Therefore, we checked the activation of UPR by the induction of a *lacZ* reporter under the control of UPRE (Fig. 4). Treatment with tunicamycin, which is a well-known inducer of UPR, clearly induced the β-galactosidase activity up to 12.8-fold in wild-type cells at 25 °C (Fig. 4A). We observed that, without any inducer, β-galactosidase activity was constitutively activated in the *gpi10-2* mutant even at the permissive temperature of 25 °C; the activity was about 13-fold higher in the *gpi10-2* mutant compared to that in the wild type. Shifting to a high temperature induced UPR in both the wild-type and *gpi10-2* mutant, β-galactosidase activity reached much higher levels in the *gpi10-2* mutant than in wild-type cells (Fig. 4B). We observed that exogenous VCP and Cdc48 overexpression decreased the β-galactosidase activity partly but not completely. These results suggested that the *gpi10-2* mutation induced UPR and that the overexpression of VCP/Cdc48 partially reduced the increased levels of UPR.

4. Discussion

In this study, we isolated a ts mutant with a mutation of *GPI10*, *gpi10-2*, which had temperature-sensitive growth defects that were rescued by VCP or Cdc48 overexpression. The *gpi10-2* mutant contained a single nucleotide change that resulted in a single amino-acid change of Gly469Glu in Gpi10 and that was located in the C-terminus region of Gpi10. The function of the C-terminus region of Gpi10 is unknown.

Currently, the mechanism of how VCP/Cdc48 expression rescued the ts phenotype of *gpi10-2*, is not clear. It is noteworthy that ubiquitin overexpression has been shown to rescue the ts mutant of *LCB1*, which encodes a serine palmitoyltransferase, the first enzyme in ceramide biosynthesis; this restores protein degradation, and reduces aggregates of the *lcb1* mutant [18]. Moreover, the maturation of GPI-anchored proteins is delayed in the *lcb1* mutant [19]. Given that VCP/Cdc48, together with some of its cofactors, functions in ubiquitin–proteasome-mediated degradation, such as ER-associated protein degradation (ERAD), it is possible that ERAD might be induced by the overexpression of VCP/Cdc48 to degrade misfolded proteins due to the lack of GPI anchors in ER, which might be the underlying cause of the ts phenotype. Consistent with this notion, *UBI4* expression also rescued the ts phenotype of *gpi10-2*, albeit partially. However, this hypothesis may be oversimplistic. We have not observed any apparent exacerbation of the growth defects, at both 25 °C and 33 °C, in double mutants of *gpi10-2* combined with either *cdc48-3*, Δ*hrd1*, Δ*doa10*, Δ*ubx2*, Δ*ubx3*, Δ*ubx4*, Δ*ubx5*, Δ*ubx6*, Δ*ubx7*, Δ*npl4*, Δ*shp1*, *ufd1-2*, or Δ*ufd3* (data not shown). At the least, these observations appear to preclude the involvement of ERAD in the ts phenotypes of *gpi10-2*. Therefore, we do not know, at present, where and how VCP/Cdc48 works to rescue the ts phenotype of *gpi10-2*.

Even with the full recovery of the growth ability of *gpi10-2* by the expression of VCP or Cdc48, this condition could only partially reduce the increased UPR, and it was not able to rescue its increased sensitivities to cell-wall stressors. Both defective phenotypes were probably due to the defective formation of GPI-anchor proteins, which was expected from the defectiveness of the mannosyl transferase activity in the *gpi10-2* mutant. These observations suggested that the mannosyl transferase activity was defective in the *gpi10-2* mutant at 37 °C and that VCP expression did not rescue this defect at 37 °C. Notwithstanding, VCP expression rescued the growth defects of the *gpi10-2* mutant at 37 °C, indicating the possibilities that Gpi10 has uncovered cell growth-related functions that differ from mannosyl transferase activity and that these functions are rescued by VCP expression. These possibilities remain to be clarified.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.01.017.

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plasmid name	vector/gene	Source/Reference
p002	pRS316-GPD	Takata et al
p003	pRS316-GPD-VCP	Takata et al
p004	pRS316-GPD-CDC48	Takata et al
p005	pRS316-UBI4(WT)	This study
p006	pRS316-GPI10(*gpi10-2*)	This study
p007	pRS316-GPI(WT)	This study
p008	pRS316-GPI10*(gpi10-2)*	This study
M123	pRS316-Gal-VCP	This study
p28-1	YEp13-MRF1	This study
p19-2	YEp13-YLL033W,RIX7,GRC3	This study
p19-3	YEp13-YLL037W,ENT4,UBI4	This study
p19-4	YEp13-YLL033W,RIX7,GRC3,PRP19,YLL037W,UBI4	This study
p19-5	YEp13-GRC3,PRP19,YLL037W	This study
p010	pRS314-GPD	Takata et al
p011	pRS314-GPD-VCP	Takata et al

神経変性疾患と細胞死

Neurodegenerative disorders and neuronal cell death

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◎神経変性疾患の病態の根幹は神経細胞死である．これまでの解析で，遺伝的・環境的な要因で凝集しやすい蛋白質がつくられることが多くの神経変性疾患の最初のステップとなっていることが示されてきた．遺伝性神経変性疾患のひとつのカテゴリーであるポリグルタミン病の解析から，細胞内の主要なATPaseであるVCPの発症への関与が明らかになり，続いて，VCP内の一アミノ酸置換が優性遺伝性の前頭側頭葉型認知症を伴う疾患や筋萎縮性側索硬化症(ALS)の原因となっていることが明らかになった．このような疾患誘導性VCPではATPase活性が亢進していることが判明し，ATPの消費の亢進が神経変性疾患に関与している可能性が推測された．本稿ではこれまでの神経変性疾患での細胞死研究の知見を紹介するとともに，ATPの減少を抑制することで細胞死を抑制する“細胞保護療法”の可能性について議論する．

Key word：神経変性疾患，VCP，ATPase，細胞死，細胞保護療法

わが国の高齢化人口の増加とともに加齢に伴う疾患が急増している．たとえば神経系の疾患では，Alzheimer病に代表される認知症の患者の増加が著しい．2013年6月1日付けの読売新聞朝刊によると，9,000人の高齢者を対象とした大規模調査から，65歳以上の日本人の認知症の有病率は15%と推定されたそうである．中枢神経系，とくに大脳はいろいろな機能領域により構成されており，ある機能領域が障害を受けるとその機能が欠落した症状を呈する．Alzheimer病では短期記憶を担う海馬や，考えたり判断することを担う大脳皮質の神経細胞が脱落(死滅)することにより，いわゆる“認知症”の症状を示す．一方，Parkinson病では黒質のドパミンニューロンが選択的に脱落し，筋萎縮性側索硬化症(ALS)では脊髄のモーターニューロンが選択的に脱落する．その結果，Parkinson病では振戦・寡動・筋硬直といった症状が，ALSでは筋肉を動かすことができなくなる．

このような神経細胞が脱落することが主要な原因となっている疾患群を総称して神経変性疾患とよんでいる．神経変性疾患は症状があまりにも多岐にわたるため，ながらく統一的な発症機構は想定されていなかったが，上記のように，共通して神経細胞の脱落が主要な原因であること，さらに遺伝性の場合，他の疾患群ではほとんど認められることがない優性遺伝形式をとる疾患が数多く存在するなどの共通性があり，これらの神経変性疾患にかなり似通った発症機構が存在する可能性が考えられた．また，Huntington病に代表されるいくつかの優性遺伝性の神経変性疾患では，つぎの世代で症状が重篤化する場合があることが認められ，この現象は“表現促進現象”とよばれていた．しかし，その実体はまったく不明であった．

遺伝性神経変性疾患に認められる優性遺伝の正体

優性遺伝病ではひとつの変異したアレルが疾患を引き起こすため，その変異はつくられる蛋白質の機能を奪う(loss-of-function)ものではなく，何らかの悪い機能を付加(toxic gain-of-fucntion)していると推測されていた．1990年代に入り優性遺伝を示すHuntington病やMach-

表 1　ポリグルタミン病の原因遺伝子の特徴

疾患	遺伝形式	染色体上の位置	遺伝子	CAG リピートの数	
				健常人	患者
球脊髄性筋萎縮症(SBMA)	XR	Xq11-12	アンドロゲン受容体	7〜34	38〜68
Huntington 病(HD)	AD	4p16.3	*HD*	10〜35	37〜121
脊髄小脳失調症 1 型(SCA1)	AD	6p23	*SCA1*	6〜39	43〜82
歯状核赤核淡蒼球ルイ体萎縮症(DRPLA)	AD	12p13	*DRPLA*	5〜35	49〜85
Machado-Joseph 病/脊髄小脳失調症 3 型(MJD/SCA3)	AD	14q32.1	*MJD1*	13〜44	65〜84
脊髄小脳失調症 2 型(SCA2)	AD	12q24.1	*SCA2*	14〜31	35〜59
脊髄小脳失調症 6 型(SCA6)	AD	19q13	α1A Ca^{2+}チャネル	4〜16	21〜27
脊髄小脳失調症 7 型(SCA7)	AD	3p12-13	*SCA7*	7〜17	38〜130
脊髄小脳失調症 17 型(SCA17)	AD	6p27	TATA 結合蛋白質	25〜42	47〜63

XR：伴性劣性遺伝形式，AD：常染色体優性遺伝形式．

ado-Joseph 病(MJD)などの原因遺伝子がつぎつぎと同定され[1,2]，その結果 9 つの遺伝性神経変性疾患で，原因遺伝子はそれぞれ異なっていたが，疾患の原因となる遺伝子変異は共通に，コーディング領域にある CAG リピートの数が増加することが判明した[3,4]．健常人の遺伝子の同じ場所にも数個〜40 個程度の CAG リピート数が存在するが，患者では正常の範囲を超えてリピート数が増加していることが示された(表 1)．しかも，CAG リピートの数が長くなるほど発症が早くなり症状が重くなっていること，つぎの世代にすこしだけリピート数が長くなって伝わる場合があり，そのとき発症年齢が早くなり症状が重篤化していることが示され，表現促進現象の遺伝子変異としての実体が解明された[3,4]．

つぎに問題となったのは，「これらの異常な CAG リピートの伸長がどのように神経変性疾患を引き起こすのか」ということであった．当時，研究が一番進んでいた Alzheimer 病で有力視されていた発症モデルに，Selkoe が提唱した“βアミロイド仮説”というものがあった[5]．“βアミロイド仮説”は剖検脳の観察から導かれたもので，まず，アミロイドβ(Aβ)が産出し，それが神経細胞外に老人斑として沈着し，続いて神経原線維変化が神経細胞内に生じ，その後，徐々に神経細胞死を誘導するというものである(図 1)．

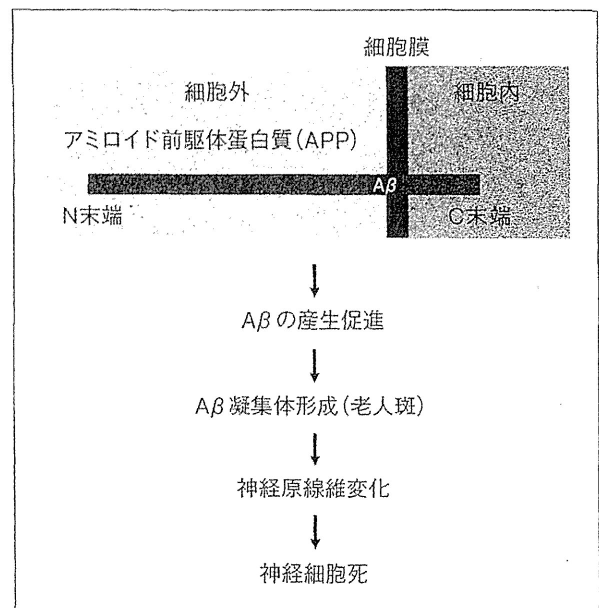

図 1　βアミロイド仮説の模式図
βアミロイド仮説ではまず，Aβが産出し，それが神経細胞外に老人斑として沈着し，続いて神経原線維変化が神経細胞内に生じ，その後，徐々に神経細胞死を誘導する．

Alzheimer 病では古くから 2 つの特徴的な病理像が観察されていた．ひとつが老人斑とよばれるもので，海馬・大脳皮質などの神経細胞のまわりの沈着物で，コンゴレッドと結合して重屈折性をし，偏光顕微鏡で観察すると青リンゴ色にみえる．もう 1 つが神経原線維変化という細胞内の異常な蛋白質の線維状の蓄積物で，現在では過剰にリン酸化を受けたタウ蛋白質であることが井原らによって解明されている[6]．老人斑の正体は 1984 年，Glenner によって老人斑がグアニジン酸に溶解することが発見され，これまで困難であった老人斑の可溶化によって，その主要構成成分が同定決定された[7]．主要成分は 40 個前後のペプチドであることが判明し，Aβと命名された．その後，Aβは一型の膜蛋白質の膜貫通蛋白質の一部であ

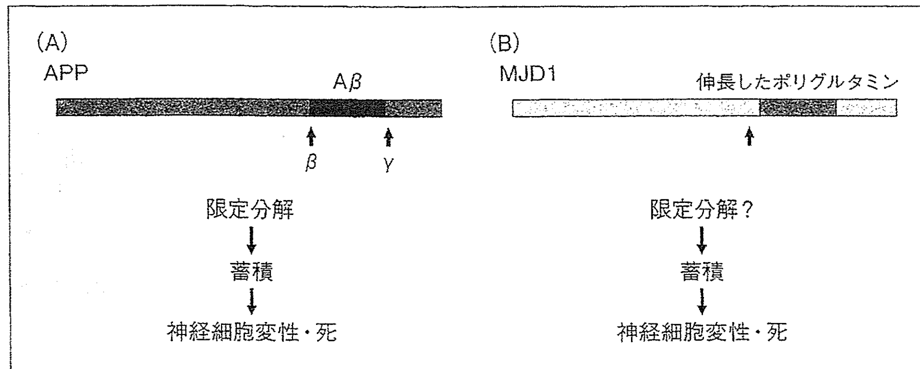

図 2　Toxic fragment hypothesis
βアミロイド仮説(A)と同様，毒性をもつペプチドフラグメントの産出を疾患発症の最初のステップとするモデルで，ポリグルタミン病の発症においてポリグルタミンを含むペプチドフラグメントの切出しを最初のステップと想定したモデル(B).

ることが判明し，その膜蛋白質はamyloid precursor protein(APP)と命名された(図1).

著者は，MJDでも同じようなことが起こってポリグルタミンが切り出されて毒性がでているという可能性を思いついた(図2)．さっそく，培養細胞にMJD蛋白質由来の伸長したグルタミンリピート(ポリグルタミン)を培養細胞に発現させてみると，細胞死が誘導されることがわかった[8]．また，蛋白質に翻訳されない伸長したCAGリピートの発現はなにも引き起こさなかった．続いて小脳Purkinje細胞にポリグルタミンを発現させるトランスジェニックマウスを作製すると，小脳が極度に萎縮し，小脳失調症の表現型が観察された[8]．これらのデータはポリグルタミンが神経変性の起因物質であることを示しており，著者らはHuntington病やMJDといったグルタミンをコードするCAGリピート数が伸長することで発症する遺伝性神経変性疾患を“ポリグルタミン病”とよぶことを提唱した[8].

Parkinson病はAlzheimer病に続いて多い神経変性疾患で，罹病率は1,000人に1人程度である．傷害を受ける中脳の黒質に残存するドパミンニューロンにはLewy小体とよばれる蓄積物をもつことが，Parkinson病の病理学的な特徴となっている．1997年，優性遺伝性のParkinson病の原因遺伝子としてαシヌクレイン遺伝子が同定された[9]．同定された2家系の変異は，それぞれA30P，A53Tという一アミノ酸置換であった．2004年，別の一アミノ酸置換E46Kをもつ家系が同定された[10]．これらのParkinson病の家系では，障害されるドパミンニューロン内にLewy小体が顕著に観察されることが報告された.

このことをヒントにSpillantiniらは，αシヌクレインの蓄積こそがLewy小体の本体であろうと考え，孤発性Parkinson病患者の死亡脳の病理解析を行った．その結果，ほぼすべてのLewy小体が抗αシヌクレイン抗体で見事に染色された[11]．また，優性遺伝性のALSにおいても，最初に同定された原因遺伝子産物である変異SOD1蛋白質(この場合も一アミノ酸置換)が，傷害を受けている脊髄前角部の運動神経細胞内に凝集体を形成していることが知られていた[12]．これらの結果は，遺伝性神経変性疾患に認められる優性の性質，すなわちtoxic gain-of-functionの本体は凝集性の高い蛋白質がつくりだされ，それらが細胞の内外で凝集・蓄積することであると総括的に考えることができる．その結果，神経細胞が変性に陥り脳から脱落し，症状として表れているのである.

神経変性疾患の発症に関与するVCP

神経変性疾患に認められる異常蛋白質の凝集物，たとえばポリグルタミンの凝集物の意義として，凝集物自体が直接的に細胞に障害を与えたり重要な細胞内因子をトラップして細胞毒性を果たすとの考えがある．著者らはこれらの考えとは別に，異常蛋白質の蓄積・凝集に対して生体反応が起こり，それが行きすぎた状態が神経変性を引き起こしているという立場で研究を進めてきた．こ

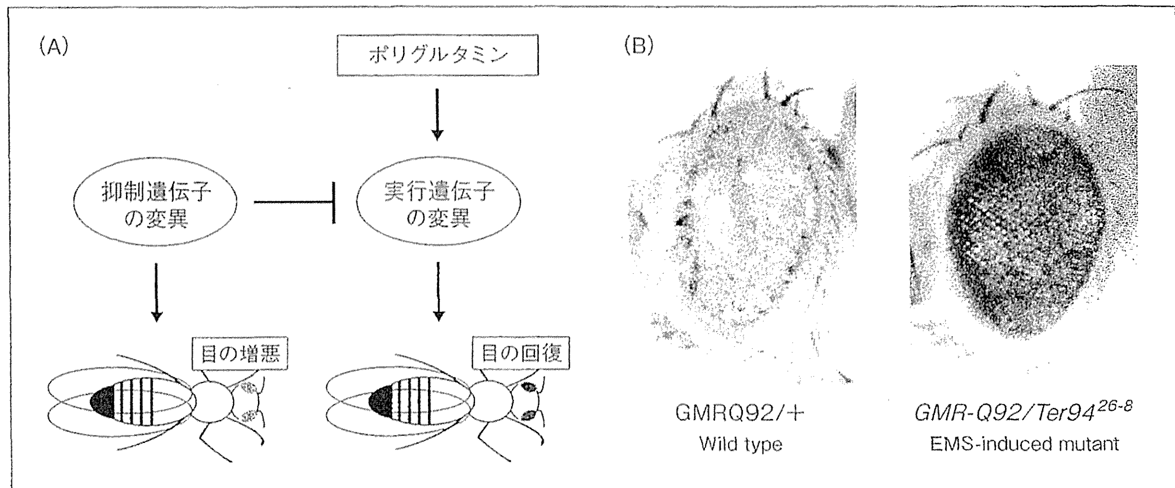

図 3　ポリグルタミンの神経細胞死に関与する遺伝子のスクリーニング法とその結果
GMR プロモーターを用いてショウジョウバエの複眼にポリグルタミンを発現させると，複眼の変性(神経細胞死)が引き起こされる．ショウジョウバエの機能低下変異体をかけあわせて複眼の変性が抑制される遺伝子を同定することで，本来の機能として神経細胞死にかかわる可能性のある遺伝子を同定することができる(A)．このスクリーニングによって，Ter94 の変異体で，ポリグルタミン(Q92)が引き起こす複眼の変性が劇的に緩和された(B)．(文献[13]より改変引用)．

の考えに基づくと，神経細胞には神経細胞死の実行に深く関与する遺伝子が存在するはずということになる．

この想定される，細胞死に関与する遺伝子の候補をみつける方法として，ショウジョウバエを用いた遺伝学的なスクリーニングを行った(図 3)．その結果，ポリグルタミンが引き起こす複眼変性(神経細胞死)にかかわる遺伝子として *Ter94* 遺伝子(ドロソフィラの VCP)を同定した(図 3)[13]．この変異体では *Ter94* 遺伝子のプロモーター領域に変異があり，Ter94 蛋白質の発現量の低下が観察された．一方，VCP 内の一アミノ酸置換により inclusion body myopathy with Paget disease of bone and frontotemporal dementia(IBMPFD)という骨格筋・心筋・骨・神経が傷害を受けるヒトの優性遺伝病が引き起こされる[14]．2010 年には同様な一アミノ酸置換が優性遺伝性の家族性 ALS を引き起こすことが報告された[15]．著者らの解析で，これらの病気を引き起こす変異 VCP は，解析したすべてで ATPase 活性が亢進していることが判明した[16]．さらに，これらの変異 VCP は単独でショウジョウバエの複眼に発現させても何も表現型を示さなかったが，ポリグルタミンと共発現させるとポリグルタミン単独の場合に比べて顕著に複眼の変性を増強した[16]．すなわち，IBMPFD に同定された変異 VCP は，異常蛋白質と共存したとき(ストレス時)に細胞の変性を増強する活性があることが示唆された．

VCPのATPase活性を阻害する化合物の開発

上記の知見は VCP の ATPase 活性を抑制することの有用性を示唆している．VCP は細胞のいろいろな機能，たとえば cell cycle，膜融合，プロテアゾームへのユビキチン化された蛋白質の運搬，小胞体で不要になった蛋白質の分解(endoplasmic reticulum-associated degradation：ERAD)などにかかわる重要な蛋白質で，培養細胞で VCP のノックダウンや優勢抑制体の強発現を行うと細胞死が誘導されるため[17]，毒性をもたない VCP 阻害剤の開発は不可能であると考えられていた．実際，VCP 阻害剤として販売されている DBeQ という化合物(IC_{50}=～1 μM)を細胞培養液に添加すると数 μM の濃度で細胞死を誘導する[18]．しかし著者らは，VCP 内の主要な ATPase 領域に隣接する D2α 領域とよばれる VCP に特異的な領域のアミノ酸置換によって，毒性をもたない ATPase 活性の失活変異体を作成しうることを報告していた[19]．これは，VCP の ATPase 活性を阻害することはかならずしもノックダウンや優勢抑制体の強

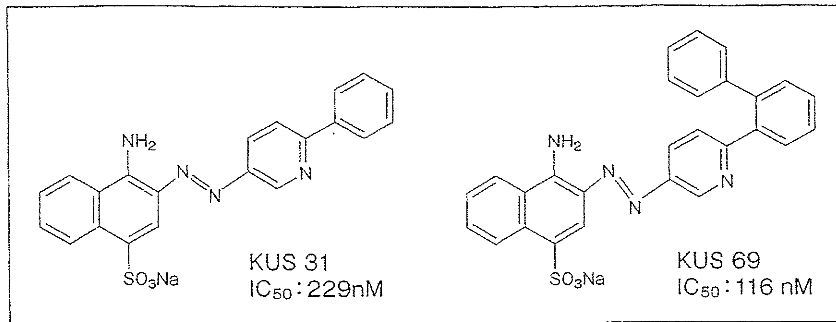

図 4　VCPのATPase活性を阻害するKUS化合物

発現と同等ではないことを示していた．そこで，リコンビナントVCPのATPase活性の抑制を指標に既存化合物をスクリーニングし，IC_{50}が1 μM以下で，数μMの濃度で培養細胞液に添加しても毒性を示さない化合物を得ることができた．この化合物をリード化合物とし，約200種類の新規化合物を合成し，Kyoto University substance (KUS)と命名した(図4)．

いくつかのKUSは100〜300 nMのIC_{50}で，VCPのATPase活性を阻害するにもかかわらず，50 μM以上の濃度で細胞培養液に添加しても細胞増殖の停止やユビキチン化蛋白質の蓄積といった，VCP機能の抑制を示す表現型はみられなかった．一般に，“ATPaseの機能はATPの加水分解によって得られるエネルギーを利用して発揮される”と信じられている．しかし，著者らが得た結果は“VCPの機能にはATPase活性はかならずしも必要ではない”ことを強く示唆した．それどころか，KUSは細胞死を誘導するような種々のストレス下(低グルコース，血清除去など)で，容量依存的に細胞死を抑制する細胞保護作用が観察された．低グルコースや血清除去は細胞内のATP量の減少を引き起こす．これらの結果と，VCPが細胞の可溶性蛋白質の約1%を占める豊富な蛋白質であることを考え合わせ，KUSの処理によってATPの減少が抑制されるに違いないという考えに至った．実際に，これらの処理によって引き起こされるATPの減少をKUSが抑制することを見出した．さらに，*in vivo*で，細胞死を主たる表現型とする緑内障および網膜色素変性症のマウスモデルにKUSを投与すると，顕著な細胞死抑制作用が観察された．

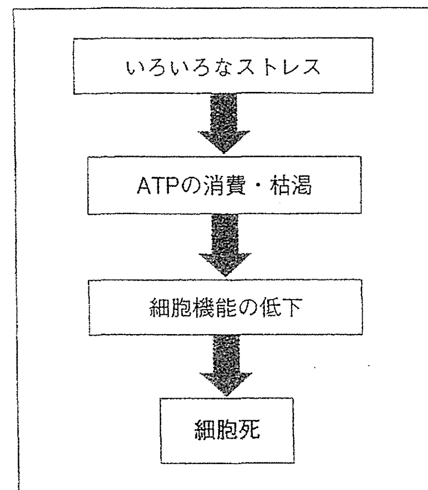

図 5　細胞死の原因としてストレスによって消費されるATPの枯渇を想定したモデル

ATPの減少抑制による細胞の保護

神経変性疾患の病態の根幹は神経細胞死であり，神経細胞死をいかに防ぎ，遅らせるかが，もっとも重要な治療戦略として位置づけられる．しかし，古くはBDNFなどの神経栄養因子から最近のcaspase阻害剤まで，生体内で神経細胞死を防ごうとする試みはことごとく失敗し，現在，まったくの手詰まり状態となっている．著者らの開発したVCPのATPase阻害剤(VCPの機能阻害剤ではない)は細胞内のATPの減少を抑制することで，細胞を細胞死から保護する作用を示した．これらのことから，細胞死誘導のあらたなモデルとして図5のモデルを考えている．すなわち，ストレス反応の多くはATPを消費する結果となり，最終的にはATPの枯渇をきたす．そして，そのようなATPの枯渇が細胞死を誘導するというモデルである．神経変性疾患では，異常蛋