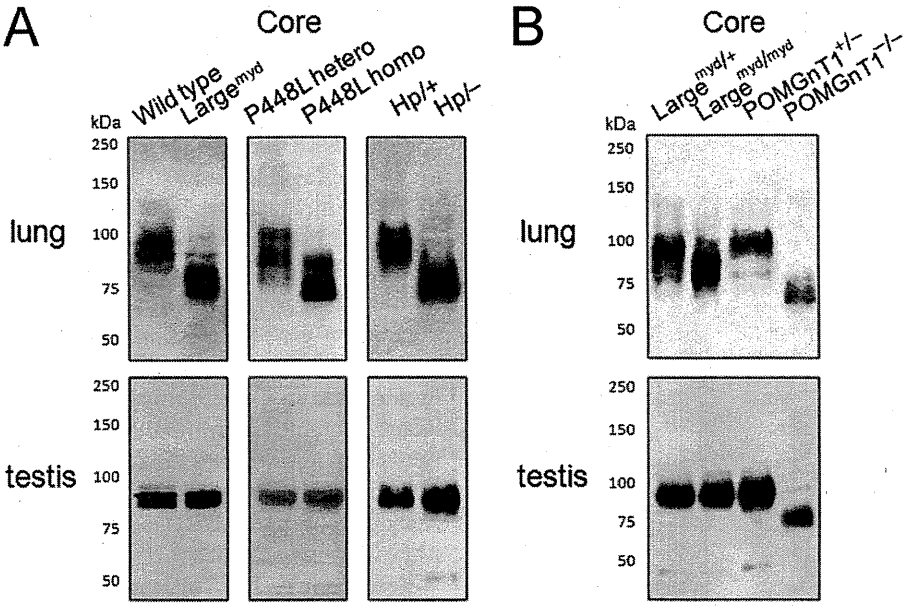

FIGURE 3. **α-DG in lung and testis from dystroglycanopathy models.** *A*, α-DG was enriched from lung and testis of wild-type, *Large*^{myd}, FKRP-P448L heterozygous or homozygous mice, and knock-in mice with a human retrotransposal allele and an intact mouse *fukutin* allele (Hp/+) or *fukutin*-deficient mice (Hp/−), and then analyzed by Western blot. *B*, the molecular weight of α-DGs from lung and testis of *POMGnT1*-deficient mice (*POMGnT1*$^{-/-}$) was compared with those of litter heterozygous mice (*POMGnT1*$^{+/-}$) and *Large*-deficient mice (*Large*$^{myd/myd}$).

(12). Our data add new evidence that mutations in *FKRP* also result in the absence of the post-phosphoryl moiety. It remains unclear how defects in *Large*, *fukutin*, *POMGnT1*, or *FKRP* all result in the same loss of the post-phosphoryl modification. A possible explanation is that these proteins may form a complex or be sequentially activated to create the post-phosphoryl moiety. POMGnT1 catalyzes GlcNAc transfer to *O*-mannose, and thus, it may not have direct involvement in the synthesis of the post-phosphoryl structure; however, the defects in post-phosphoryl modification in *POMGnT1*-deficient cells or tissues, shown here and in another study (12), indicate that the GlcNAc-β1,2 branch on *O*-mannose might provide favorable circumstances for the post-phosphoryl modification. Together, these studies have suggested that recognition by IIH6 requires at least the post-phosphoryl structure on *O*-mannose.

The range of α-DG molecular size and its reactivity to the monoclonal antibody IIH6 varies widely among different tissues. This has been thought to result from tissue-specific glycosylation on α-DG (1, 30). Our results indicate that post-phosphoryl modification is tissue-specific and thus suggest that the difference is largely determined by the extent and/or the proportion of post-phosphoryl modification. In light of the lack of post-phosphoryl modification in normal tissues such as lung and testis, even in the presence of transcripts of all known genes responsible for α-DG glycosylation, possible explanations are that they may not be properly translated; their protein products may be inactive (*e.g.* improper cellular location and lack of modification); or protein levels are not sufficient for α-DG glycosylation. Another possibility is that there could exist other yet-to-be identified mechanisms for α-DG modification, for example, a negative regulator, or novel genes. Supporting this idea, a large-scale genetic study has indicated that almost half of dystroglycanopathy cases can be explained by unidentified disease-causative genes or factors (32). Some of these cases might be caused by mutations in unidentified disease-causative genes, whose products are involved in post-phosphoryl modification, and such genes might not be expressed in wild-type tissues lacking post-phosphoryl modification of α-DG. This situation is exemplified in studies using cancer cells. It has been reported that several malignant cancer cell types lose the laminin-binding glycan of α-DG due to epigenetic down-regulation of *LARGE* or defects in the LARGE-binding protein β3GnT1, raising the possibility of defects in post-phosphoryl modification of α-DG in those cells (31, 33).

Reduction or loss of IIH6 reactivity can be rescued by forced expression of LARGE (34, 35). It has been shown that exogenously expressed LARGE can overcome defects in the laminin-binding activity of α-DG in *fukutin*- or *POMGnT1*-deficient cells or tissues (26, 34). On the other hand, if cells lack a gene that acts via direct interaction with LARGE, such as β3GnT1, forced expression of LARGE would fail to produce IIH6 reactivity (31). We observed that forced expression of LARGE could produce IIH6 reactivity in CHL cells, and newly produced IIH6-reactive α-DG no longer bound to IMAC-beads (supplemental Fig. 1). The effect of LARGE overexpression on α-DG glycosylation was also observed in TM3 cells. These data indicate that CHL and TM3 cells might lack gene activity that is involved in the post-phosphoryl modification, but such defects can be compensated by overexpression of LARGE.

Our results also raised a question about the function of the non-laminin-binding form of α-DG. It is generally thought that α-DG function relies on its glycosylation-dependent laminin-binding activity; on the other hand, several studies have suggested that dystroglycan possesses functions beyond that of a laminin receptor. The N-terminal domain of α-DG, which can be shed from the core protein into the extracellular space and body fluid (36), has been shown to promote neurite extension in PC 12 cells, suggesting that it has a biological function (37).

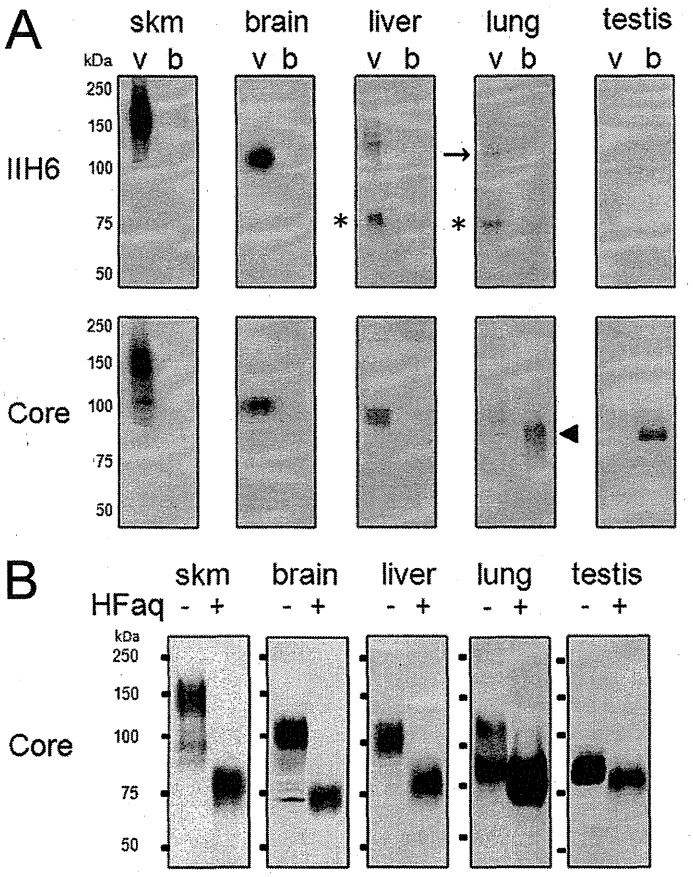

FIGURE 4. **Absence of post-phosphoryl modification in wild-type lung and testis.** *A*, IMAC bead-binding assays for α-DG from wild-type tissues. α-DG-enriched samples from skeletal muscle (*skm*), brain, liver, lung, and testis of C57BL/6 mice were tested for binding to IMAC beads. The void (*v*) and bound (*b*) fractions were collected. The *arrow* indicates the IIH6-positive population of lung α-DG. The *arrowhead* indicates the IIH6-negative fraction of lung α-DG bound to beads. An *asterisk* indicates a background signal that is not specific for IIH6 antibody. *B*, chemical dephosphorylation of α-DG from wild-type tissues. α-DG-enriched samples from skeletal muscle (*skm*), brain, liver, lung and testis of C57BL/6 mice were treated with HFaq and then analyzed by Western blot using anti-DG core antibody.

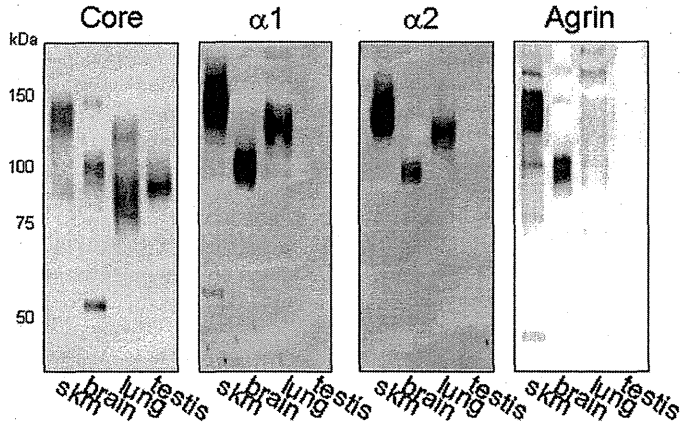

FIGURE 5. **Ligand-binding assays for lung and testis α-DG.** Ligand binding (laminin α1, α2, and agrin) was assessed in α-DG-enriched samples from skeletal muscle (*skm*), brain, lung, and testis using ligand overlay assays.

α-DG might have ligand proteins that do not require *O*-mannosyl modification; for example, a chondroitin sulfate proteoglycan biglycan has been shown to interact with protein core of the α-DG C-terminal domain in a glycosylation-independent

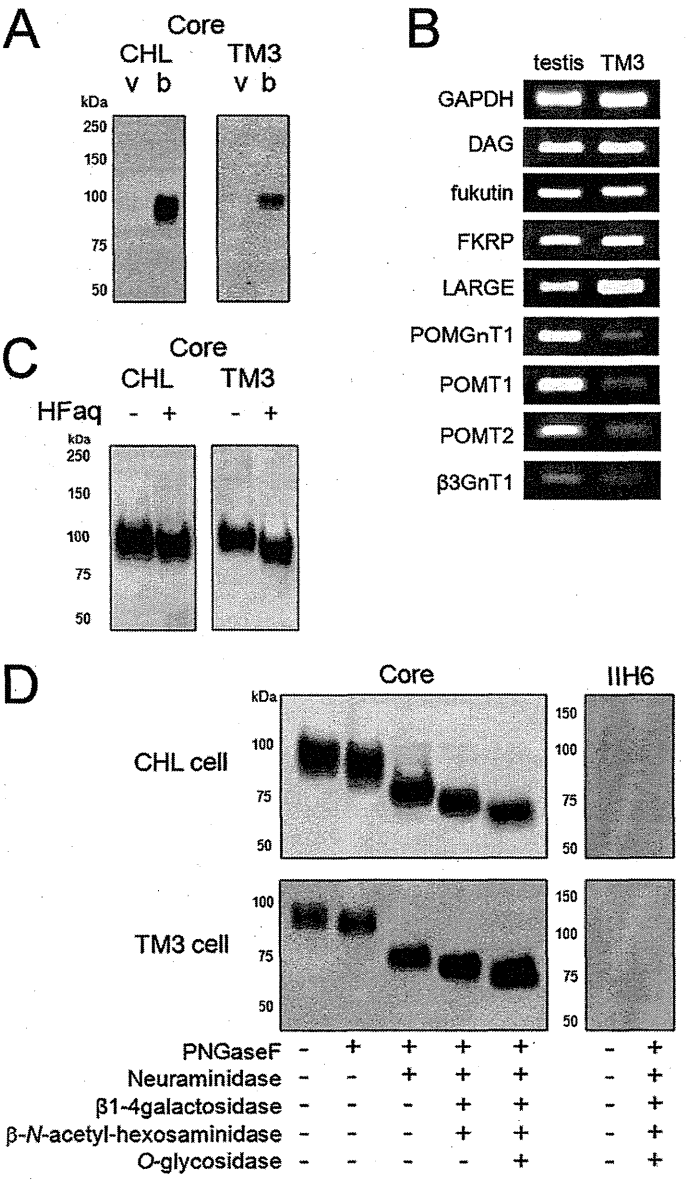

FIGURE 6. **Absence of post-phosphoryl modification of α-DG in CHL and TM3 cells.** *A*, IMAC bead-binding assays for α-DG from CHL cells and TM3 cells. α-DG-enriched samples from CHL and TM3 cell lysates were tested for binding to IMAC beads. The void (*v*) and bound (*b*) fractions were collected. *B*, RT-PCR analysis of TM3 cells. RT-PCR analysis was performed to detect transcripts encoding proteins implicated in α-DG glycosylation. *C*, chemical dephosphorylation of α-DG from wild-type tissues. α-DG-enriched samples from CHL and TM3 cell lysates were treated with HFaq. *D*, enzymatic deglycosylation of α-DG from CHL and TM3 cells. α-DG-enriched samples from CHL and TM3 cells were digested with glycosidase mixtures (peptide-*N*-glycosidase (*PNGaseF*), neuraminidase, β1–4 galactosidase/β-*N*-acetyl-hexosaminidase, and *O*-glycosidase). Following the IMAC bead-binding assay, HFaq treatment, and enzymatic deglycosylation, the samples were analyzed by Western blot, using antibodies against the α-DG core protein (Core) or the functionally glycosylated form (IIH6).

manner (38). DG is also thought to serve as a signaling molecule (39). For example, the cytoplasmic tail of β-DG interacts with several signaling molecules, including caveolin-3, Grb2, and mitogen-activated protein (MAP) kinase kinase 2 (40). Although the significance of these interactions is not well understood, it is possible that DG serves as a scaffold to position interacting proteins at their proper cellular location (9, 41). Taken together, these observations suggest that the presence of

DG without post-phosphoryl modification could be functionally important in various tissue types.

Future work to determine the molecular structure of the post-phosphoryl moiety, and to identify genes involved in its biosynthesis, will contribute to understanding the biological basis of this unique post-translational modification and disease pathogenesis. Our present data contributes to the foundation for such research. Recently, it has been shown that LARGE can act as a bifunctional glycosyltransferase, with both xylosyltransferase and glucuronyltransferase activities (42). Involvement of these activities in the post-phosphoryl modification also should be clarified in the future.

Overall, our results indicate that phosphorylated *O*-mannose not only plays critical roles in the pathogenesis of dystroglycanopathy but also is a key determinant in the maturation of α-DG as a laminin receptor in normal tissues and cells.

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Mislocalization of Fukutin Protein by Disease-causing Missense Mutations Can Be Rescued with Treatments Directed at Folding Amelioration*[S]

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Background: The molecular pathogenesis of fukutin-deficient dystroglycanopathy remains unclear, and no effective treatment is available.
Results: Some disease-causing missense fukutin mutants showed mislocalization in cultured cells, which can be corrected by treatments directed at folding amelioration.
Conclusion: Correction of cellular localization of disease-causing mutants may have a therapeutic benefit.
Significance: A possible therapeutic strategy for fukutin-deficient dystroglycanopathy is proposed based on its molecular pathogenesis.

Fukuyama-type congenital muscular dystrophy (FCMD), the second most common childhood muscular dystrophy in Japan, is caused by alterations in the *fukutin* gene. Mutations in *fukutin* cause abnormal glycosylation of α-dystroglycan, a cell surface laminin receptor; however, the exact function and pathophysiological role of fukutin are unclear. Although the most prevalent mutation in Japan is a founder retrotransposal insertion, point mutations leading to abnormal glycosylation of α-dystroglycan have been reported, both in Japan and elsewhere. To understand better the molecular pathogenesis of fukutin-deficient muscular dystrophies, we constructed 13 disease-causing missense *fukutin* mutations and examined their pathological impact on cellular localization and α-dystroglycan glycosylation. When expressed in C2C12 myoblast cells, wild-type fukutin localizes to the Golgi apparatus, whereas the missense mutants A170E, H172R, H186R, and Y371C instead accumulated in the endoplasmic reticulum. Protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) also mislocalizes when co-expressed with these missense mutants. The results of nocodazole and brefeldin A experiments suggested that these mutant proteins were not transported to the Golgi via the anterograde pathway. Furthermore, we found that low temperature culture or curcumin treatment corrected the subcellular location of these missense mutants. Expression studies using *fukutin*-null mouse embryonic stem cells showed that the activity responsible for generating the laminin-binding glycan of α-dystroglycan was retained in these mutants. Together, our results suggest that some disease-causing missense mutations cause abnormal folding and localization of fukutin protein, and therefore we propose that folding amelioration directed at correcting the cellular localization may provide a therapeutic benefit to glycosylation-deficient muscular dystrophies.

Fukuyama-type congenital muscular dystrophy (FCMD,[2] MIM 253800) is the second most common childhood muscular dystrophy and one of the most prevalent autosomal recessive disorders in the Japanese population. FCMD is clinically characterized by congenital muscular dystrophy in combination with cortical dysgenesis (micropolygyria) and ocular abnormalities (1). We identified *fukutin*, the gene responsible for FCMD, on chromosome 9q31 by linkage analysis and positional cloning (2, 3). Most FCMD-bearing chromosomes have been derived from a single ancestral founder and have a 3-kb retrotransposal insertion in the 3′ noncoding region of the *fukutin* gene. Compound heterozygosity, with both a retrotransposal mutation and a point mutation in *fukutin*, is sometimes seen and generally exhibits more severe pathologies (4, 5). However, a recent report has identified several Japanese patients presenting with mild limb-girdle dystrophy (LGMD2M, MIM 611588) and normal intelligence (6) and who have a retrotransposal mutation and a point mutation in the *fukutin* gene. Outside Japan, *fukutin* mutations have been reported in patients with various phenotypes, from Walker-Warburg syndrome (WWS, MIM 236670) to LGMD (7–13). Overall, the current common understanding is that *fukutin* alterations can give rise to a wide spectrum of phenotypes.

Mutations in *fukutin* cause abnormal glycosylation of the cell surface laminin receptor α-dystroglycan (DG) and reduce its

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[S] This article contains supplemental Tables I–III, Experimental Procedures, and Figs. 1–3.
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[2] The abbreviations used are: FCMD, Fukuyama-type congenital muscular dystrophy; BFA, brefeldin A; DG, dystroglycan; ER, endoplasmic reticulum; FKRP, fukutin-related protein; LGMD, limb-girdle muscular dystrophy; POMGnT1, protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1; POMT1, protein *O*-mannosyltransferase 1; POMT2, protein *O*-mannosyltransferase 2; WWS, Walker-Warburg syndrome.

laminin binding activity (14). The α- and β-DG complex is believed to provide physical strength to the sarcolemma by connecting the basal lamina to the cytoskeleton. Thus, abnormal glycosylation caused by *fukutin* mutations underlies FCMD molecular pathogenesis, but the exact function of fukutin remains unclear. The *fukutin* gene encodes a 461-amino acid protein with a predicted molecular mass of 53.7 kDa (3). Although endogenous fukutin protein has not been detected in cells, likely due to its low abundance, expression studies have proposed that *fukutin* gene product localizes to the Golgi apparatus (15, 16). Fukutin protein contains a transmembrane domain (3, 16), a putative *N*-glycosylation site (3), and a DxD motif that is predicted to modify cell surface glycoproteins or glycolipids (17). Previously, we showed that the transmembrane domain of fukutin binds to the protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1), which is encoded by the responsible gene for muscle-eye-brain disease (MIM 253280) (18), suggesting that fukutin affects the enzymatic activity of POMGnT1 (16). Other regions of the fukutin protein share no sequence homology with known proteins. In addition to FCMD, several other forms of muscular dystrophy are caused by abnormal glycosylation of α-DG; together, these conditions are termed as "dystroglycanopathy." To date, six genes (protein *O*-mannosyltransferase 1 (*POMT1*), protein *O*-mannosyltransferase 2 (*POMT2*), *POMGnT1*, *fukutin*, fukutin-related protein (*FKRP*), and *LARGE*) have been implicated in dystroglycanopathies, and all are thought to be involved in glycosylation of α-DG (19–23). POMGnT1 and the POMT1/2 complex are known to have glycosyltransferase activities directly involved in synthesis of *O*-mannosyl sugar chains on α-DG (18, 24). Quite recently, it has been shown that LARGE can act as a bifunctional glycosyltransferase with both xylosyltransferase and glucuronyltransferase activities (25). On the other hand, the exact function of FKRP is unknown. Yoshida-Moriguchi *et al.* reported that a phosphodiester-linked moiety on *O*-mannose of α-DG is defective in LARGE- or fukutin-deficient dystroglycanopathies (26). This finding suggests that LARGE and fukutin might be involved in the synthesis of the postphosphoryl modification, which is necessary for laminin binding activity.

The precise pathogenic mechanism of FCMD has remained obscure. In this report, to understand molecular pathogenesis of fukutin-deficient muscular dystrophies, we constructed 13 disease-causing missense *fukutin* mutations that have been reported inside and outside Japan (4–6, 9–13, 27, 28) and investigated their pathological roles in fukutin intracellular location. Four mutants (A170E, H172R, H186R, and Y371C) lost their Golgi localization and instead accumulated in the endoplasmic reticulum (ER) when expressed in C2C12 cultured cells. Using *fukutin*-null mouse embryonic stem (ES) cells, we showed that these mutants retain the activity responsible for α-DG glycosylation. Finally, we found that low temperature culture and curcumin treatment are effective in correcting the localization of these missense fukutin mutants.

EXPERIMENTAL PROCEDURES

Reagents—Brefeldin A (BFA) and nocodazole were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Curcumin was purchased from Nacalai Tesque (Kyoto, Japan).

Antibodies used in this study were as follows: monoclonal anti-V5 (Invitrogen), rabbit polyclonal anti-FLAG (Sigma), monoclonal anti-α-DG clone IIH6C4 (Millipore), monoclonal anti-β-DG clone 8D5 (Novocastra Laboratories, Newcastle, UK), monoclonal anti-GM130 (BD Biosciences), monoclonal anti-KDEL antibodies (Stressgen, Victoria, Canada); rabbit polyclonal anti-laminin (Sigma); and goat polyclonal antibody against the C-terminal region of α-DG (AP-074G-C) (29)

Vector Constructions and Site-directed Mutagenesis—For the construction of expression vectors, the coding regions of human *POMGnT1*, human *fukutin*, or human *FKRP* with a FLAG or a V5 epitope at the C terminus were cloned into the pEF1/V5-HisA vector (Invitrogen). Expression vectors encoding 13 different disease-causative missense fukutin mutants were constructed using site-directed mutagenesis. Mutations were confirmed by DNA sequencing.

Cell Culture, Transfection, and Immunofluorescence Detection—Mouse myoblast C2C12 cells were cultured in Dulbecco's modified Eagle Medium (DMEM) (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and streptomycin (100 μg/ml) (Wako). Mouse ES cells were grown in DMEM with 15% heat-inactivated fetal bovine serum, 100 μM 2-mercaptoethanol, and streptomycin. Targeted disruptions of the *fukutin* gene in ES cells have been described previously (30).

Cell transfection was performed using Effectene (Qiagen) according to the manufacturer's protocol. 48 h after transfection, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and then permeabilized in PBS with 0.5% Triton X-100 (Nacalai Tesque). For immunofluorescence detection, after blocking with 1% BSA (Wako) in PBS at room temperature for 1 h, the cells were first incubated for 90 min with polyclonal anti-FLAG, monoclonal anti-GM130, monoclonal anti-KDEL, or monoclonal anti-V5 antibodies, followed by Alexa Fluor 488-conjugated anti-rabbit IgG and/or Alexa Fluor 546-conjugated anti-mouse IgG (Invitrogen) for 1 h at room temperature. After a final rinse with PBS, cells were observed by fluorescence microscopy using a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany).

For BFA, nocodazole, or curcumin treatment, the cells were incubated with 5 μg/ml BFA or 10 μg/ml nocodazole for 2 h after 48 h of transfection, or 10 μg/ml curcumin for 24 h after 24 h of transfection. For statistical analysis of fukutin cellular localization, cells expressing fukutin were classified into four classes (Golgi localization, Golgi and around localization, dot localization, and ER localization) (see Fig. 6*A*). For statistical analysis of POMGnT1 localization, cells co-expressing fukutin/POMGnT1 were classified into three classes (Golgi localization with fukutin, Golgi localization without fukutin, and ER localization). The number of cells in each class was counted and analyzed using the χ^2 test.

Dystroglycan Preparation—DG was enriched from solubilized mouse ES cells. The ES cells were solubilized in 1 ml of PBS containing 1% Triton X-100 and protease inhibitor mixture (Nacalai Tesque). Solubilized fractions were incubated with 30 μl of wheat germ agglutinin-agarose beads (Vector Laboratories) at 4 °C for 2 h. The beads were washed five times with 1 ml of PBS containing 0.1% Triton X-100 and protease inhibitor

TABLE 1
Disease-causing missense mutations in the *fukutin* gene

Mutation	Other allele	Severity	References
A114T	T286 frame shift	Mild	10
G125S[a]	5370–5842 deletion (3′-UTR)	Severe	11
G125S[a]	F390 frame shift	Severe	11
M133T	3-kb insertion	Typical	28
A170E	Y371C	Typical	13
H172R	3-kb insertion	Typical	27
R179T	3-kb insertion	Mild	6
H186R	Homozygote	Severe	12
R246G	R47 nonsense	Mild	13
C250G	3-kb insertion	Typical	4
W305C	Homozygote	Typical	10
R307Q	Homozygote	Mild	13
R307Q	F390 frame shift	Mild	9
R307Q	N455 frame shift	Mild	9
Q358P	3-kb insertion	Mild	6
Y371C	3-kb insertion	Typical	5
Y371C	A170E	Typical	13

[a] G125S has been registered as a polymorphism (rs_34006675).

mixture, then directly boiled for 5 min in SDS-polyacrylamide gel loading buffer.

SDS-PAGE, Western Blotting, and Laminin Overlay Assay—Cell lysates were dissolved in SDS sample buffer and subjected to SDS-PAGE in 10% gels or 7.5% gels. Gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), probed with anti-FLAG, anti-α-DG core protein (AP-074G-C), anti-α-DG sugar chain (IIH6C4), or anti-β-DG antibodies, and then developed with horseradish peroxidase-conjugated secondary antibody (DAKO, Carpinteria, CA). Blots were processed using ECL plus Western blotting detection system (GE Healthcare) and exposed to Fuji RX-U x-ray film (Fuji Film, Kanagawa, Japan). The laminin overlay assay was performed according to the method of Michele *et al.* (14).

Additional methods are described under supplemental Experimental Procedure.

RESULTS

Mislocalization of Disease-causing Missense Mutant Fukutin Proteins—To examine the cellular location of fukutin proteins containing disease-causing missense mutations, we constructed expression vectors encoding wild-type or mutant fukutin proteins with a FLAG epitope at the C terminus. The missense mutants analyzed in this study have been identified inside and outside Japan (Table 1), and their clinical phenotypes vary from severe WWS-like to mild LGMD-type without mental retardation (Table 1 and Fig. 1). These constructs were transfected into C2C12 myoblast cells, and the cellular localizations of the expressed fukutin proteins were examined by immunofluorescence. Immunofluorescent signals indicated co-localization of the expressed wild-type fukutin with the Golgi apparatus marker GM130 (162/176 cells; Golgi + Golgi and around/total cells) (Fig. 2*A*, *merge*, and supplemental Table I). Nine of the 13 missense mutants (A114T (97/113), G125S (140/144), M133T (109/120), R179T (142/149), R246G (101/110), C250G (124/134), W305C (183/198), R307Q (131/134), and Q358P (174/182)) also co-localized with GM130 (supplemental Table I), indicating that these mutations do not affect the cellular location of fukutin protein. In contrast, the A170E (13/146), H172R (8/145), and H186R (6/141) mutants, as well as the previously reported Y371C (8/128) mutant (16), did not co-localize with GM130 (Fig. 2*A* and supplemental Table I), instead showing co-localization with the ER marker KDEL (Fig. 2*B*). These results indicated that A170E, H172R, H186R, and Y371C aberrantly localize to the ER.

Accumulation of Missense Fukutin Mutants in ER Caused by Impaired Transport to the Golgi—Accumulation of the four mutants in the ER might result from improper cellular trafficking. To determine whether the mutants are not properly transported from the ER to the Golgi or whether they are transported back to the ER after reaching the Golgi, we treated C2C12 cells expressing wild-type fukutin or the four mutants with nocodazole (an inhibitor for retrograde transport from the Golgi to the ER) or BFA (an inhibitor for anterograde transport from the ER to the Golgi). If the four mutants reached the Golgi and then were immediately transported back to the ER, the four mutant proteins should be detected in the Golgi after nocodazole treatment. Immunofluorescent signals indicating ER accumulation of the four mutants were observed following a 2-h incubation with 10 μg/ml nocodazole (Fig. 3*C–F*, *panels 4*). When cells expressing wild-type fukutin were incubated with 5 μg/ml BFA, the wild-type fukutin was detected in the ER (99/107; ER/total cells) (Fig. 3*B*, *panel 6*, and supplemental Table II), as seen in the cells expressing any of the four mutants. These data suggested that failure of proper transport via the anterograde pathway causes mislocalization of the four mutants to the ER.

Correction of Cellular Location of Mutant Fukutin Proteins by Low Temperature Culture—We hypothesized that mislocalization resulted from protein misfolding and therefore examined whether the localization of the four missense mutants could be corrected by folding amelioration. It has been reported that cell culture at low temperature can ameliorate folding and correct the subcellular localization of missense mutant proteins (31). In culture at 37 °C, the four missense fukutin mutants (A170E, H172R, H186R, and Y371C) were co-localized with KDEL (Fig. 4*A*). At low temperature (27.5 °C), the A170E (130/144; Golgi + Golgi and around/total cells), H172R (130/159), and Y371C (102/158) mutants preferentially co-localized with GM130 (Fig. 4*A* and supplemental Table I), indicating that the ER accumulation had decreased and proper localization to the Golgi was restored. Most of the expressed H186R mutant protein, however, remained in the ER (134/155; ER/total cells) at 27.5 °C; only a small proportion of this mutant shifted to the Golgi (Fig. 4*A* and supplemental Table I).

POMGnT1, which has been shown to interact with fukutin and localize to the Golgi (151/156; Golgi with fukutin/total cells) (supplemental Table III) (16), also mislocalized to the ER when co-expressed with the mutants A170E (89/103; ER/total cells), H172R (102/113), H186R (96/111), and Y371C (98/109) (Fig. 4*B*, 37 °C, and supplemental Table III). These results indicated that fukutin mislocalization also affects the cellular location of POMGnT1. Low temperature culture of cells expressing both POMGnT1 and any of the mutants A170E (158/173; Golgi with fukutin/total cells), H172R (103/129), or Y371C (126/192) restored POMGnT1 subcellular localization to the Golgi (Fig. 4*B*, 27.5 °C, and supplemental Table III). When expressed with the H186R mutant, the POMGnT1 localization shifted to the Golgi despite the majority of the H186R mutant remaining in

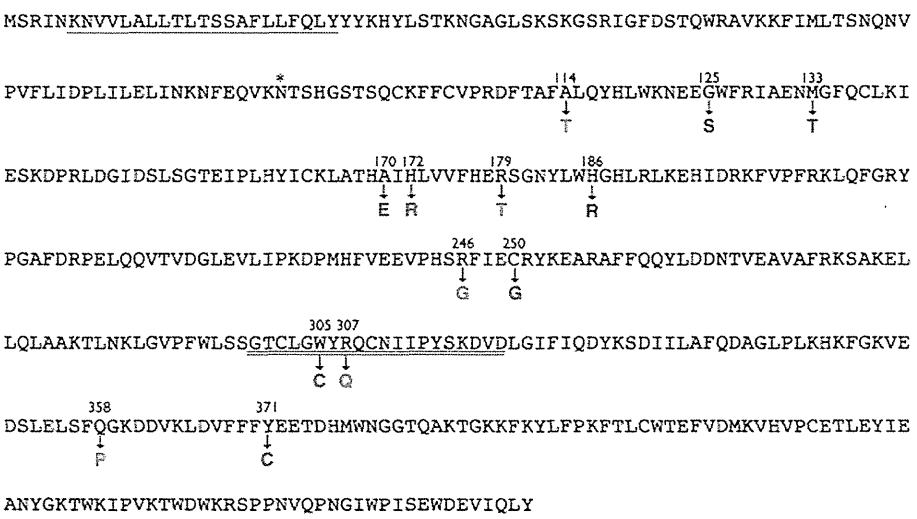


FIGURE 1. **Amino acid sequence of fukutin and location of missense mutations.** Amino acid changes are indicated along with severity of phenotype. *Blue*, mild form (CMD without mental retardation, LGMD, or cardiomyopathy). *Purple*, typical form (FCMD, CMD with mental retardation); *red*, severe form (WWS). *Single underline* indicates the transmembrane domain, the *asterisk* indicates an *N*-glycosylation site, and the *double underline* indicates the DxD motif.

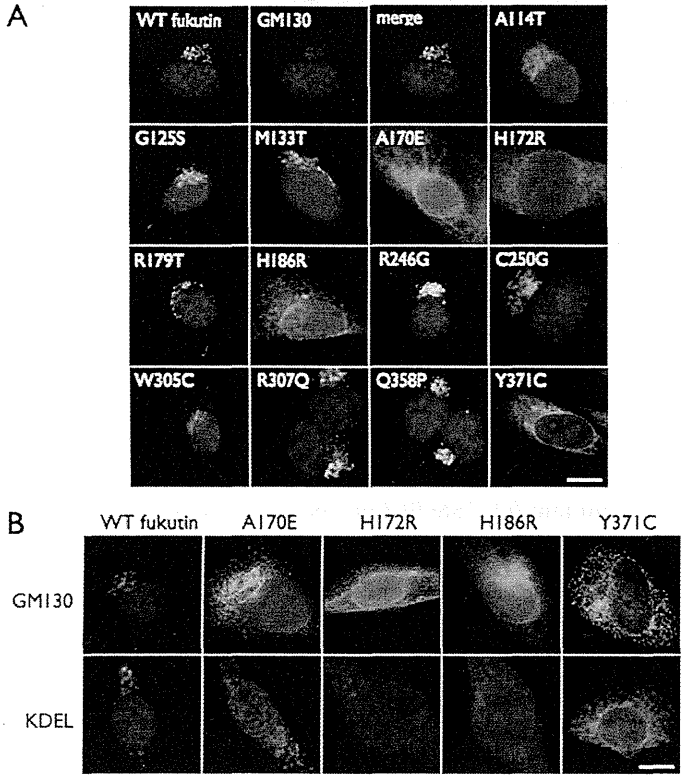


FIGURE 2. **Some disease-causing missense mutations cause abnormal cellular localization of fukutin.** *A*, localization of the FLAG-tagged wild-type or missense fukutin proteins in transfected C2C12 myoblast cultured cells. Cells were double-labeled with anti-FLAG (*green*) and a Golgi marker GM130 (*red*). No co-localization was seen for the missense fukutin mutants A170E, H172R, H186R, and Y371C. Wild-type (*WT fukutin*) and the other nine mutants co-localized with GM130. *B*, mislocalization of missense fukutin mutants to the ER. The fukutin missense mutants A170E, H172R, H186R, and Y371C (*green*) lost their localization to the Golgi (*red*) in transfected C2C12 cultured cells (*upper*). Co-localization of these mutants with the ER marker KDEL (*red*) was seen in the cultures (*lower*). *Blue*, DAPI, indicating the nucleus. *Scale bars*, 10 μm.

the ER (108/141; Golgi without fukutin/total cells) (Fig. 4*B* and supplemental Table III). These data showed that low temperature culture could correct mislocalization of the mutant fukutin proteins, but its effect may depend on the position of missense mutations within the protein.

Residual Function of Fukutin Missense Mutants to Restore α-Dystroglycan Glycosylation—C2C12 cells contain endogenous fukutin, making it difficult to evaluate the α-DG modification activity of exogenously expressed mutant fukutin proteins. Instead, we used *fukutin*-null mouse ES cells (FCMDp182 cell) (30) to examine whether the four missense mutant fukutin proteins retain the activity responsible for α-DG modification. We expressed the four mutants (A170E, H172R, H186R, and Y371C) in FCMDp182 cells (supplemental Fig. 1) and then examined the recovery of glycosylation and laminin binding activity of α-DG. In mock-transfected FCMDp182 cells cultured at either 37 °C or 27.5 °C, α-DG showed no detectable reactivity against the monoclonal IIH6C4 antibody, which recognizes the functionally glycosylated form of α-DG. We also observed hypoglycosylation of α-DG, as indicated by lower molecular mass, and no α-DG laminin binding activity in mock-transfected cells (Fig. 5, *lanes 2* and *10*). When wild-type fukutin was expressed in FCMDp182 cells, we observed restoration of the laminin binding activity and the IIH6C4 reactivity of α-DG, but at much weaker levels than for α-DG in the wild-type mouse ES cell line AB2.2 (*lanes 1* and *3*). This partial restoration and the faint amount of α-DG core protein bands around 100 kDa (the size of α-DG in the AB2.2 cells) may result from low transfection efficiency. Expression of each of the four missense mutants in the FCMDp182 cells restored the IIH6C4 reactivity and the laminin binding activity to levels comparable with those observed in the wild-type fukutin transfectants (*lanes 3–7*). Although these missense mutants preferentially localized to the ER at 37 °C, it is possible that small amounts of the expressed mutants reached the Golgi. To support this interpretation, a report has shown that the mislocalized mutant protein ΔF508 cystic fibrosis transmembrane conductance regulator, which is the most common mutation leading to cystic fibrosis, shows partial function when overexpressed (32). We have not detected restoration of the IIH6C4 reactivity and the laminin binding activity by expression of a mutant fukutin protein with direct substitutions in the DxD motif (D317A/V/D319A) (supplemental Fig. 2). Expression of FKRP, a member

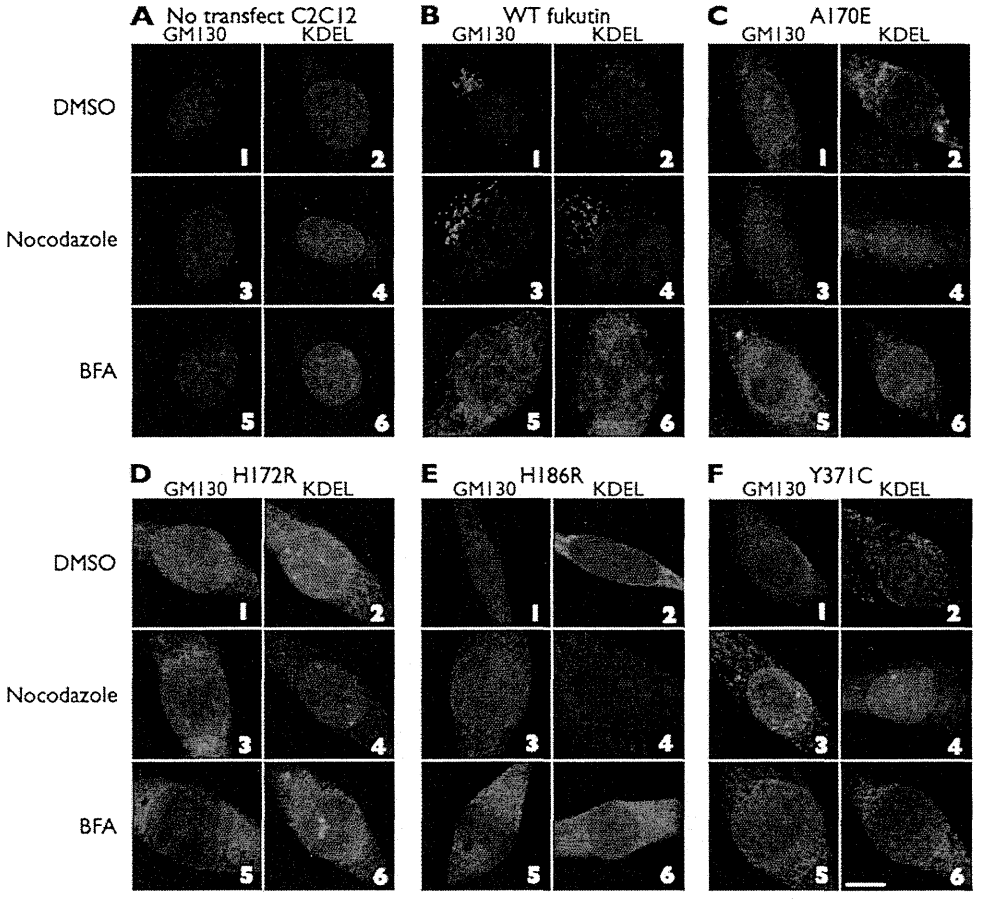


FIGURE 3. **Accumulation of the missense mutants in the ER could be caused by the failure of proper transport via the anterograde pathway.** C2C12 cells without (*A*) or with expression of wild-type fukutin (*B*) or the missense mutants (*C–F*) were incubated with nocodazole or BFA. Nocodazole treatment did not change the cellular localization of the expressed mutant fukutin proteins, whereas BFA treatment shifted wild-type fukutin to the ER. *Red*, GM130 or KDEL; *green*, expressed fukutin proteins; *blue*, DAPI. *Scale bar*, 10 μm.

of the fukutin protein family, in FCMDp182 cells showed no effect on α-DG glycosylation (*lane 8*). We also performed these experiments at low culture temperature (27.5 °C) and obtained similar results (Fig. 5, *lanes 9–16*). The reason for reduced efficiency of glycosylation recovery in low temperature culture is unclear, but the low temperature might affect enzymatic activities involved in the α-DG glycosylation pathway. Because the expression level of each mutant protein was different (Fig. 5, anti-FLAG), it was not possible to simply compare their residual activity. Importantly, however, all four mutants retained the activity responsible for α-DG modification.

Correction of Mislocalization of Missense Fukutin Mutants by Curcumin Treatment—Our results indicated that mislocalization of some missense mutants could cause disease-related abnormal glycosylation of α-DG. Therefore, we next searched for chemicals that could restore proper localization. Curcumin, a nontoxic natural constituent of turmeric spice, has shown the ability to correct misfolding and mislocalization of the ΔF508 mutant (33). We incubated C2C12 cells expressing the four mutants (A170E, H172R, H186R, or Y371C) with 10 μg/ml curcumin at 37 °C for 24 h. Among the four mutants, the A170E mutant showed the greatest benefit from curcumin treatment. In the absence of curcumin, approximately 90% of the cells showed ER localization of the A170E mutant (159/175; ER/total cells), and only a few cells (7/175; Golgi + Golgi and around cells) showed Golgi localization (Fig. 6). Curcumin treatment significantly decreased the ER mislocalization signal of the A170E mutant (69/156; ER/total ells) and increased the Golgi- and Golgi/around signals (72/156; Golgi + Golgi and around cells) ($p = 8.28 \times 10^{-20}$). Although not as dramatic as seen with A170E, the other mutants showed slight beneficial changes in their cellular distributions following curcumin treatment (Fig. 6*B* and supplemental Table II). We also examined glycerol, arginine, and 17-allylaminogeldanamycin for beneficial effects on localization of the mutants, but none was observed (data not shown).

DISCUSSION

In this report, we have demonstrated that some disease-causing missense fukutin mutants lost their Golgi localization in C2C12 cultured cells and that this mislocalization can be corrected by low temperature culture or curcumin treatment. We identified four missense mutants that localized abnormally to the ER. Nocodazole treatment did not alter their ER localization, and low temperature culture shifted three of the four mutants to the Golgi. It is generally recognized that cell culture at low temperature can ameliorate protein misfolding and correct abnormal cellular localization (31). Therefore, we presume that some missense mutants could not be transported to the Golgi via the anterograde pathway because of protein misfold-

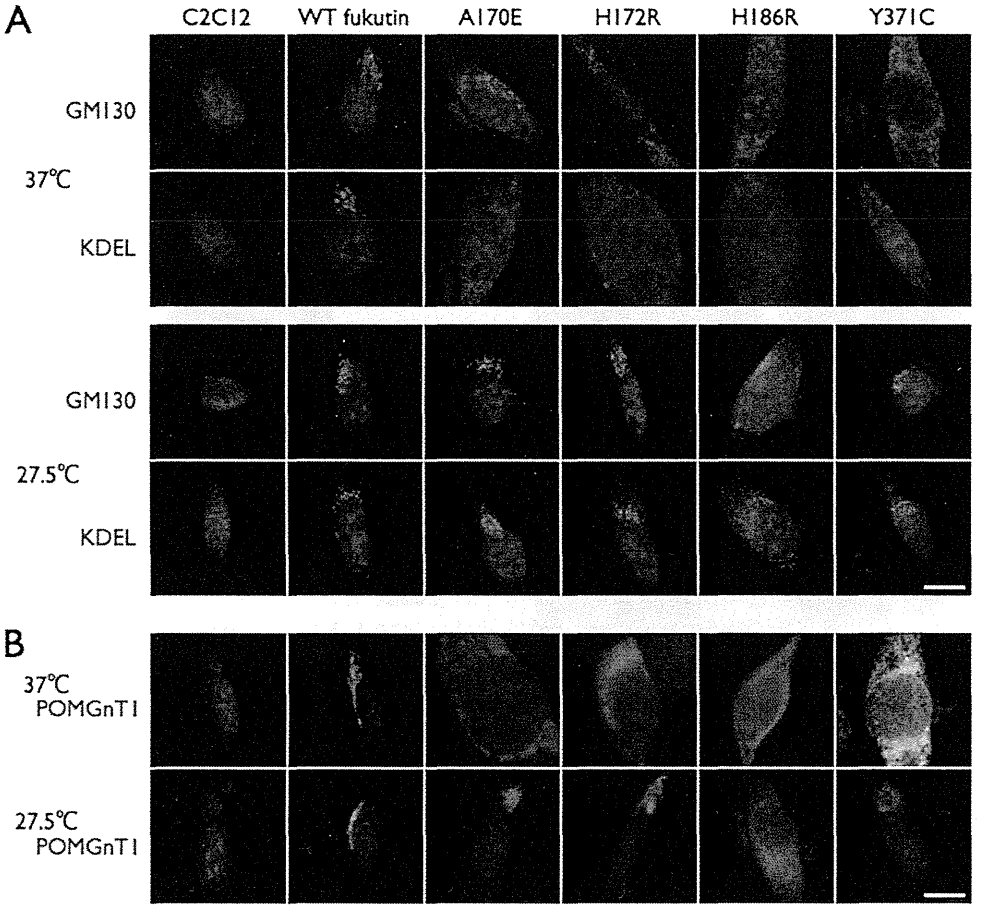


FIGURE 4. **Low temperature culture corrects mislocalization of mutant fukutin proteins.** *A*, effects of low temperature culture on the localization of the missense fukutin mutants. At 37 °C, the missense fukutin mutants (A170E, H172R, H186R, and Y371C, *green*) co-localized with KDEL (ER marker, *red*) in C2C12 cultured cells (*upper*). In contrast, at 27.5 °C, the missense fukutin mutants A170E, H172R, and Y371C lost their co-localization with KDEL and acquired co-localization with GM130 (Golgi marker, *red*) (*lower*). A large amount of the mutant H186R protein retained the ER localization in 27.5 °C culture. *B*, POMGnT1 localization when co-expressed with the missense mutants. POMGnT1 (*red*) mislocalized to the ER when co-expressed with the missense fukutin mutants (A170E, H172R, H186R, or Y371C, *green*) at 37 °C. However, at 27.5 °C, localization of POMGnT1 was restored to the Golgi even when co-expressed with mutant fukutin proteins. *Blue*, DAPI. *Scale bars*, 10 μm.

ing caused by amino acid substitutions. A large amount of the fourth mutant protein, H186R, retained the ER localization even under low temperature conditions (Fig. 4*A*). It has been reported that the patient with the homozygous H186R mutation is affected with WWS (12), which shows more severe pathological features than typical FCMD. The H186R mutation in the *fukutin* gene may affect protein folding severely enough that low temperature conditions cannot correct mislocalization. We reported previously that POMGnT1 interacts with fukutin and co-localizes to the Golgi (16). Our present data show that correction of the mislocalization of the three missense fukutin mutants by low temperature culture was accompanied by correction of POMGnT1 cellular localization (Fig. 4*B*). This POMGnT1 behavior is rational because the transmembrane region of fukutin, through which fukutin binds to POMGnT1 (16), remained intact in these missense fukutin mutants (Fig. 1, *single underline*). Immunoprecipitation experiments confirmed the interaction between each of the missense fukutin mutants and POMGnT1 (supplemental Fig. 3). Most of the H186R mutant remained in the ER at 27.5 °C, but POMGnT1 expressed with the H186R localized to the Golgi. The reason for this result is uncertain, but a possible explanation is that the H186R mutant may have a harmful (dominant negative-like) effect on POMGnT1 localization, and this effect may be ameliorated at low temperature.

The above-mentioned four mutants, which showed abnormal localization to the ER, were identified in patients presenting with a typical or a severe phenotype. However, several of the remaining nine mutants, which showed Golgi localization, were also identified in patients presenting with the typical or the severe phenotype (G125S, M133T, C250G, and W305C) (Table 1). Therefore, the typical or severe phenotypes seem not always to be related to abnormal cellular localization of mutated proteins. Given that FCMD is inherited in an autosomal recessive manner, disease-causing mutations in the *fukutin* gene must lead to loss of function of the fukutin protein. Although the exact function of fukutin is undetermined, these nine mutations may disrupt an important functional domain in the protein. For example, the W305C (10) and R307Q (9, 13) mutations are located in the DxD motif (Fig. 1, *double underline*), which is predicted to be involved in the modification of cell surface glycoproteins or glycolipids (17). These substitutions may disrupt the DxD motif and produce dysfunctional fukutin protein. Five mutations (A114T, R179T, R246G, R307Q, and Q358P) have been identified in patients presenting with mild phenotypes (congenital muscular dystrophy with no

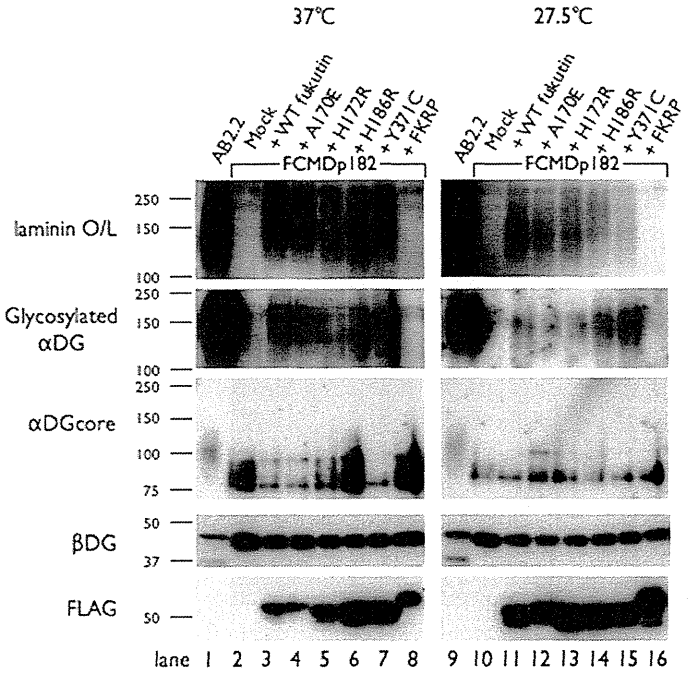
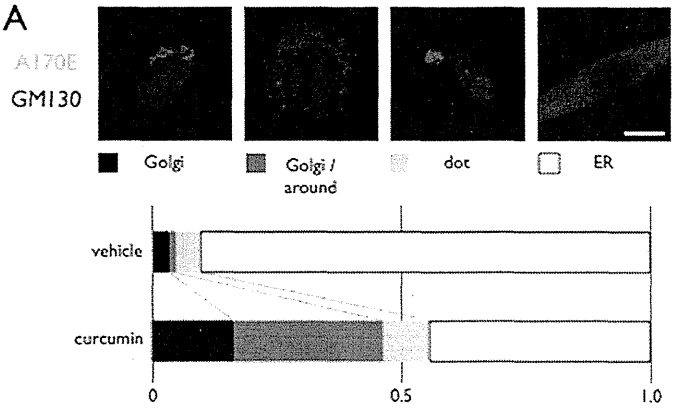


FIGURE 5. **Fukutin missense mutants retain the activity responsible for α-DG glycosylation.** Western blot analysis was performed to detect IIH6C4 (glycosylated α-DG), core α-DG, β-DG, and FLAG tag, and the laminin overlay assay. DG and fukutin proteins were prepared from wild-type mouse ES cells (AB2.2), fukutin-deficient mouse ES cells (FCMDp182), and fukutin- or FKRP-transfected FCMDp182 cells, and cultured at 37 °C and 27.5 °C. FCMDp182 cells expressing any of the mutant fukutin proteins showed levels of glycosylated α-DG signal (IIH6C4-reactivity) and laminin binding activity that were comparable with those observed in FCMDp182 cells expressing wild-type fukutin.



B

	A170E		H172R		H186R		Y371C	
	vehicle	curcumin	vehicle	curcumin	vehicle	curcumin	vehicle	curcumin
Golgi	6	25	1	0	0	0	0	0
Golgi / around	1	47	1	32	0	32	1	11
dot	9	15	1	5	1	5	11	11
ER	159	69	109	102	113	158	115	114
p value	8.28×10^{-20}		1.10×10^{-6}		0.005		0.02	

FIGURE 6. **Curcumin treatment partly corrects mislocalization of the missense fukutin mutants.** *A*, effects of curcumin treatment on the cellular localization of the missense A170E fukutin mutants. C2C12 cells expressing the missense fukutin mutant A170E were cultured in the presence or absence of 10 μg/ml curcumin. Cells were classified into four classes (Golgi localization, Golgi and around localization, dot localization, and ER localization), then counted and statistically analyzed using the χ^2 test. *Red*, GM130; *green*, expressed missense fukutin protein A170E; *blue*, DAPI. *Scale bar*, 10 μm. *B*, statistical analysis of effects of curcumin treatment on the cellular localization of the missense fukutin mutants A170E, H172R, H186R, and Y371C.

mental retardation, LGMD with no mental retardation, or cardiomyopathy) (6, 9, 10, 13). In the present study, these five mutant proteins were localized to the Golgi when expressed in C2C12 cells. In patients with R179T or Q358P mutations, α-DG shows residual reactivity against the monoclonal antibody IIH6C4, which recognizes functionally glycosylated α-DG (6), indicating that these fukutin mutations retain partial function in the DG maturation pathway.

It is of interest that some missense fukutin mutants retain α-DG glycosylation activity and that their mislocalization could be partly corrected by treatments directing at folding amelioration. These observations suggest that drugs capable of correcting the localization might have therapeutic benefits in patients who carry these missense fukutin mutants. Although approximately half of the cells expressing the A170E mutant retained the ER accumulation signals following curcumin treatment (Fig. 6), recent studies have indicated that even partial restoration of α-DG glycosylation can produce therapeutic effects (29). Efforts to identify more efficient folding amelioration reagents may lead to therapeutic strategies.

A large number of missense mutations have been identified in dystroglycanopathy. It has been reported that missense mutations in *POMT1* and *POMGnT1* compromise enzymatic activity in the gene products (18, 34, 35). Disease-causing missense mutations in *POMT2*, *LARGE*, and *FKRP* have been also identified (10, 21–23, 36, 37). Recently, Kawahara *et al.* have reported that expression of some disease-causing missense FKRP mutant proteins in FKRP knock-down zebrafish restores α-DG glycosylation, suggesting a residual FKRP function in these missense mutants (38); however, phenotype improvement depends on the location of the mutation. The FKRP missense mutations C318Y and A455D, which failed to improve the fish phenotype in the report from Kawahara *et al.* (38), were reported to show abnormal cellular localization when expressed in certain cell lines (39). On the other hand, using several different cell lines, Dolatshad *et al.* suggested that a reduced protein (putative enzymatic) function of FKRP rather than protein mislocalization is the primary mechanism of disease (39). Interestingly, Bao *et al.* have reported cells lacking FKRP transcripts but expressing IIH6C4-reactive α-DG (40). This finding may indicate a possibility of a FKRP-independent glycosylation pathway. Alternatively, only a subtle amount of FKRP, even below detectable level by RT-PCR, may be sufficient for α-DG glycosylation. This implies that some missense mutants can restore IIH6C4-reactive α-DG if only a little protein function remains. Of the increasing number of identified disease-causing missense mutations, some may alter the cellular location of the protein, which can be a direct cause of disease. Correction of cellular localization or folding amelioration may have a therapeutic benefit for dystroglycanopathies caused by missense mutations, although the finding from cell culture experiments must be interpreted cautiously when extrapolating to human disease.

In this study, we have used curcumin to correct mislocalization of missense fukutin mutants. It has been reported that curcumin can correct misfolding and mislocalization of cystic fibrosis transmembrane conductance regulator with the ΔF508 mutation (33). Curcumin has also been reported to have protective effects in neurodegenerative diseases by inhibiting protein misfolding and aggregation in Creutzfeldt-Jakob disease and Parkinson disease (41, 42). These studies indicate that curcumin or its derivatives might be new candidate compounds for protein-folding diseases. In addition, the use of pharmacological chaperones to stabilize or promote correct folding of mutant proteins has been shown as a potential therapeutic approach to phenylketonuria, in which more than 500 disease-causing mutations have been identified (43). Our results contribute to a deeper understanding of the molecular pathogenesis of fukutin-deficient muscular dystrophy and have led us to propose a novel therapeutic strategy directed at correction of cellular localization and/or folding amelioration of disease-causing missense mutant proteins.

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Comprehensive Research Synopsis and Systematic Meta-Analyses in Parkinson's Disease Genetics: The PDGene Database

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Abstract

More than 800 published genetic association studies have implicated dozens of potential risk loci in Parkinson's disease (PD). To facilitate the interpretation of these findings, we have created a dedicated online resource, PDGene, that comprehensively collects and meta-analyzes all published studies in the field. A systematic literature screen of ~27,000 articles yielded 828 eligible articles from which relevant data were extracted. In addition, individual-level data from three publicly available genome-wide association studies (GWAS) were obtained and subjected to genotype imputation and analysis. Overall, we performed meta-analyses on more than seven million polymorphisms originating either from GWAS datasets and/or from smaller scale PD association studies. Meta-analyses on 147 SNPs were supplemented by unpublished GWAS data from up to 16,452 PD cases and 48,810 controls. Eleven loci showed genome-wide significant ($P<5\times10^{-8}$) association with disease risk: *BST1*, *CCDC62/HIP1R*, *DGKQ/GAK*, *GBA*, *LRRK2*, *MAPT*, *MCCC1/LAMP3*, PARK16, *SNCA*, *STK39*, and *SYT11/RAB25*. In addition, we identified novel evidence for genome-wide significant association with a polymorphism in *ITGA8* (rs7077361, OR 0.88, $P=1.3\times10^{-8}$). All meta-analysis results are freely available on a dedicated online database (www.pdgene.org), which is cross-linked with a customized track on the UCSC Genome Browser. Our study provides an exhaustive and up-to-date summary of the status of PD genetics research that can be readily scaled to include the results of future large-scale genetics projects, including next-generation sequencing studies.

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¶ Memberships of the consortia are provided in Text S1.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease with a prevalence of ~1% over 60 years of age [1]. Approximately 5–10% of the patients show an autosomal dominant or recessive mode of inheritance, and several causative genes have been identified, e.g. *SNCA*, *LRRK2*, *PARK2*, and *PINK1* (for review see ref. [2]). Recently, two other novel autosomal dominant PD genes, *VPS35* and *EIF4G1* [3–5], have been identified, the former via application of next-generation sequencing techniques. It can be anticipated that causal mutations in additional genes will emerge within the next years. However, the vast majority of patients suffer from non-Mendelian forms of PD, which are likely caused by the combined effects of genetic and environmental factors. In order to decipher the genetic architecture underlying PD susceptibility, more than 800 genetic association studies have been performed over the past 20 years. While early candidate gene studies and subsequent meta-analyses provided conclusive evidence showing that polymorphisms in *SNCA* [6] (encoding alpha-synuclein), *LRRK2* [7] (leucine-rich repeat kinase 2), *MAPT* [8] (microtubule-associated protein tau), and *GBA* [9] (acid beta-glucosidase) significantly impact PD susceptibility, most association studies in the field provided inconclusive or even conflicting results.

During the last few years, genome-wide association studies (GWAS) [10–19] have postulated additional PD loci. While the early GWAS and a GWAS-meta-analysis [20] were of limited sample sizes and yielded mostly inconsistent results, more recent studies have identified a number of loci that were independently confirmed in follow-up studies (e.g. *GAK*, *BST1*, and PARK16, see Table 1 for all proposed GWAS findings across GWAS publications). Very recently, a GWAS meta-analysis [21] implicated several other new putative PD loci which currently await further validation. Despite this progress, approximately 40% or more of the population-attributable risk probably remains unexplained by today's most promising PD loci [21]. To this end, genetic association studies remain one of the mainstays of PD genetics research. However, GWAS and other large-scale association studies typically only highlight the most promising results and often do not provide data on variants showing suggestive evidence for association, or previously implied variants that could not be confirmed in the GWAS setting. As a result, the cumulative genetic evidence in favor of or against association with certain variants in the PD field is becoming increasingly difficult to follow, evaluate and interpret. To address this problem, we have comprehensively collected, catalogued and systematically meta-analyzed the data from all genetic association studies published in the field of non-Mendelian PD, including GWAS, and made all results publicly available on a regularly updated online database, "PDGene" (http://www.pdgene.org).

Results

Database content

The results of this research synopsis are based on a freeze of the PDGene database content on March 31st 2011 (available upon request from the authors). At that time, PDGene included details on 828 individual studies across more than 50 different countries and six continents reporting on 3,382 polymorphisms in 890 genetic loci. Data for more than 2,000 SNPs were supplemented by results derived from up to three publicly available GWAS datasets [10,12,13] following extensive quality control and imputation. Ultimately, this procedure yielded a total of 867 polymorphisms across ~300 genetic loci that met our criteria for meta-analysis (see Methods). Additional independent GWAS data

Author Summary

The genetic basis of Parkinson's disease is complex, i.e. it is determined by a number of different disease-causing and disease-predisposing genes. Especially the latter have proven difficult to find, evidenced by more than 800 published genetic association studies, typically showing discrepant results. To facilitate the interpretation of this large and continuously increasing body of data, we have created a freely available online database ("PDGene": http://www.pdgene.org) which provides an exhaustive account of all published genetic association studies in PD. One particularly useful feature is the calculation and display of up-to-date summary statistics of published data for overlapping DNA sequence variants (polymorphisms). These meta-analyses revealed eleven gene loci that showed a statistically very significant ($P<5\times10^{-8}$; a.k.a. genome-wide significance) association with risk for PD: *BST1*, *CCDC62/HIP1R*, *DGKQ/GAK*, *GBA*, *LRRK2*, *MAPT*, *MCCC1/LAMP3*, PARK16, *SNCA*, *STK39*, *SYT11/RAB25*. In addition and purely by data-mining, we identified one novel PD susceptibility locus in a gene called *ITGA8* (rs7077361, $P=1.3\times10^{-8}$). We note that our continuously updated database represents the most comprehensive research synopsis of genetic association studies in PD to date. In addition to vastly facilitating the work of other PD geneticists, our approach may serve as a valuable example for other complex diseases.

for 147 SNPs yielding P values of ≤ 0.1 in these initial meta-analyses were provided by researchers of all remaining currently published Caucasian GWAS datasets [13,15–19,22]. Following the identification of genome-wide significant association with an intronic SNP (rs7077361) in *ITGA8* after addition of these data, we obtained additional data from the same GWAS datasets on ~1,400 SNPs in the chromosomal region encompassing *ITGA8* (chr10:15346353–15801533, hg18). Finally, independent replication data in Caucasian and Asian populations from the GEO-PD consortium [23] generated for ten recently described PD loci [21] were made available for inclusion. As a result, we were able to substantially increase the sample size (up to 16,452 PD cases and 48,810 controls) for a large number of some of the most promising PD loci. For instance, we were able to add data from up to 48,861 previously not analyzed combined cases and controls to meta-analyses of some of the recently proposed PD loci [21] (median sample size 14,896, see Table 2 and Table S1 for details). In addition to these focused analyses, PDGene displays meta-analysis results for more than seven million additional SNPs originating from up to three publicly available GWAS datasets [10,12,13]. The results are available online (e.g. as summarized in http://www.pdgene.org/largescalemeta.asp), where they are cross-linked to a customized and fully browsable track on the UCSC Genome Browser.

PDGene meta-analysis results

The PDGene meta-analyses of the 867 core polymorphisms were based on a median of 7,680 subjects (interquartile range 4,612–16,726). Additional meta-analyses were performed after stratification for Caucasian and Asian ancestry (for details on sample size and included ethnicities for individual meta-analyses see Table S1). In addition, we also performed random-effects meta-analyses across all three publicly available GWAS datasets [10,12,13] following genotype imputation using data from the International HapMap Consortium and 1000 Genomes Project. Ultimately this yielded 7,123,920 SNPs that could be meta-analyzed across at least two GWAS datasets (see Figure S1 for a quantile-to-quantile plot of the GWAS-only meta-analyses). All 867 core meta-analysis results are available online on PDGene as forest plots, summarizing the relative contributions of each dataset to the most current summary effect estimate, and in the form of cumulative plots, illustrating how summary ORs evolve over time. All meta-analysis results are plotted in Figure 1 (green dots) alongside the GWAS-only meta-analysis results (black and grey dots).

One-hundred-three meta-analyses across 12 genetic loci (*BST1*, *CCDC62/HIP1R*, *DGKQ/GAK*, *GBA*, *ITGA8*, *LRRK2*, *MAPT*, *MCCC1/LAMP3*, PARK16, *SNCA*, *STK39*, *SYT11/RAB25*) yielded summary ORs suggesting a genome-wide significant ($P\leq5\times10^{-8}$) increase or decrease in PD risk in all ethnicities and/or after stratification for ethnic ancestry (Table 2, Table S1, and Figure S2 [forest plots]). None of these loci contained more than one SNP independently associated at genome-wide significance (as judged by pair-wise linkage disequilibrium assessments using 'SNAP' and r^2-values of 0.2 as cut off http://www.broadinstitute.org/mpg/snap/). The majority of polymorphisms tested in the genome-wide significant loci do not show evidence for publication bias (Table S1). Finally, all genome-wide significant signals were robust against potential undetected sample overlap using a recently proposed procedure [24] (see Table S2 for more details). Combined sample sizes for all 12 loci were substantially larger here as compared to any previously published meta-analysis (Table S1), providing unequivocal evidence for an involvement of these loci in PD susceptibility. While power to detect genome-wide significance was excellent for most of these loci (>80% based on an OR of 1.15, and a minor allele frequency down to 0.05 using the Genetic Power Calculator, http://pngu.mgh.harvard.edu/~purcell/gpc/), power was less for a large number of other meta-analyses due to smaller sample sizes and allele frequencies (see Table S1 for details). Thus, no simple statistic can summarize the overall power of our study.

The above list includes an intronic polymorphism in *ITGA8* located on chromosome 10p13 for which we identified novel evidence for genome-wide association with PD risk (OR 0.88, $P=1.3\times10^{-8}$, $I^2=0$, see Table 2, and Figure 2). This SNP had previously been proposed to be associated with PD risk at sub-genome-wide significance by Simon-Sanchez et al [13]. After obtaining and meta-analyzing GWAS data from ~1,400 additional SNPs in this region derived from all Caucasians GWAS datasets [10,12,13,15–19,21,22], rs7077361 remained the most significantly associated SNP in this region (Figure S3).

In addition to using random-effects models, we also performed exploratory fixed-effect meta-analyses on all eligible polymorphisms. These analyses did not reveal genome-wide significant effect sizes for any additional locus, except *ACMSD/TMEM163* (most significant SNP rs6723108, OR 0.91, $P=1.3\times10^{-9}$, $I^2=46\%$ [95% CI 0–73%], Figure S4, panel 1) and *HLA* (most significant SNP chr6:32609909, OR 0.78, $P=8.8\times10^{-15}$, $I^2=84\%$ [95% CI 70–91%], Figure S4, panel 2), both of which were reported to be associated with PD risk at genome-wide significance in previous work [16,21]. In both instances, the lack of genome-wide significance in the random-effects models (Table S1) was due to relatively pronounced heterogeneity of effect estimates across studies. However, the heterogeneity across the 11 datasets in the *ACMSD/TMEM163* meta-analysis is almost entirely due to variance of effect size estimates in the same direction (see Figure S4, panel 1), making it likely that *ACMSD/TMEM163* represents a genuine PD risk locus. For the SNP tested in the *HLA* locus (chr6:32609909, Figure S4, panel 2), heterogeneity is more pronounced and more complex owing to ORs on either side of

Table 1. Overview of genome-wide association studies (GWAS) published in PD until March 31, 2011.

GWAS	Design GWAS (Follow-up)	Population GWAS (Follow-up)	# SNPs	# PD GWAS (Follow-up)	# CTRL GWAS (Follow-up)	"Featured" genetic loci
Maraganore, 2005 (ref. 9)	Family-based (case-control)	USA-LEAPS (USA)	198,345	443 (332)	443 (332)	*CDCP2, GALNT3, GWA_2q36.3, GWA_4q28.1, GWA_4q28.3, GWA_5p15.32, GWA_7p14.2, GWA_10q21.1, PASD1, PRDM2, SEMA5A*
Fung, 2006 (ref. 10)	Case-control (-)	USA-NINDS	408,803	267 (-)	270 (-)	*BRDG, DLG2, GLT25D2, GWA_4q35.2, GWA_7p12, GWA_10q11.21, GWA_11q11, GWA_16q23.1, GWA_22q13, NEGR1, ULK2, ZNF313*
Pankratz, 2009 (ref. 11)	Case-control (-)	USA-PROGENI/GenePD (-)	328,189	857 (-)	867 (-)	***DGKQ/GAK, GPRIN3, MAPT, SNCA***
Simon-Sanchez, 2009 (ref. 12)	Case-control (case-control)	USA-NINDS, Germany (USA, Germany, UK)	463,185	1,745 (3,452)	4,047 (4,756)	***LRRK2, MAPT,*** **PARK16,** ***SNCA***
Satake, 2009 (ref. 13)	Case-control (case-control)	Japan (Japan)	435,470	1,078 (993)	2,628 (15,753)	***BST1, LRRK2,*** **PARK16,** ***SNCA***
Edwards, 2010 (ref. 14)	Case-control (-)	USA-HIHG (-)	491,376	604 (-)	619 (-)	***MAPT, SNCA***
Hamza, 2010 (ref. 15)	Case-control (-)	USA-NGRC (-)	811,597	2,000 (-)	1,986 (-)	***GAK/DGKQ,*** *HLA* locus, ***MAPT, SNCA***
Spencer, 2011 (ref. 16)	Case-control (case-control)	UK-WTCCC2 (France)	1,733,533	1,705 (1,039)	5,175 (1,984)	***BST1, GAK/DGKQ, MAPT,*** **PARK16,** ***SNCA***
Saad, 2011 (ref. 17)	Case-control (case-control)	France (UK-WTCCC2, Australia)	492,929	1,039 (3,232)	1,984 (7,064)	***BST1,*** *GWA_12q24,* ***SNCA***
Simon-Sanchez, 2011 (ref. 18)	Case-control (case-control)	Netherlands	514,799	772 (-)	2024 (-)	***BST1,*** *HLA* locus, ***GAK/DGKQ, MAPT, SNCA***

The overview is based on content on the PDGene website (http://www.pdgene.org; current on March 31st, 2011). Studies are listed in order of publication date. '# PD GWAS' and '# CTRL GWAS' refers to sample sizes used in the initial GWAS datasets, whereas 'Follow-up' refers to the total number of replication samples where applicable. 'Featured genes' are those genes/loci that were declared as 'associated' in the original publication; note that criteria for declaring association varies across studies. Genetic loci in bold font denote genes showing genome-wide significant results ($P<5\times10^{-8}$) in the PDGene meta-analyses.
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1. This could be due to a number of reasons, e.g. subtle and uncorrected population substructure and/or different LD patterns between the analyzed SNP and the actual functional variant(s) [16]. Thus, although the evidence is currently not as conclusive as for *ACMSD/TMEM163* it still appears quite possible that there is one or more PD association signals in the *HLA* region. Regardless of these considerations, additional data are needed to more firmly assess the role of both loci in contributing to PD susceptibility.

Ethnicity-specific meta-analysis results

SNCA, *LRRK2*, *BST1*, and PARK16 show evidence for genome-wide significance in meta-analyses restricted to Caucasian and Asian populations (Table 2). Furthermore, data obtained from the GEO-PD consortium [23] suggest that the effect estimates for some of the recently discovered PD loci (i.e. *CCDC62/HIP1R*, *MCC1*, and *STK39*) [21] may be comparable in Caucasian and Asian populations (Table S1), although additional datasets are needed to establish genome-wide significance in populations of Asian-descent for these loci. Conversely, only insufficient data are currently available to assess the effect sizes of *GAK* and *SYT11/RAB25* on PD risk in Asians: *GAK* rs6599388 violated Hardy-Weinberg equilibrium in Asian datasets from the GEO-PD consortium and was thus excluded from further analyses on that ethnic group [23]. *SYT11/RAB25* chr1:154105678 was excluded from all analyses due to technical reasons in the study by the GEO-PD consortium [23]. Moreover, none of the reported *SYT11/RAB25* and *GAK* SNPs from the recent GWAS meta-analysis [21] were captured directly or by proxy (with an $r^2\geq0.8$) in the Japanese GWAS dataset [14,23]. Finally, Asian-descent populations cannot be appropriately assessed for PD association with the *MAPT*-H1/H2 haplotype, rs10928513 in *ACMSD*, and rs7077361 in *ITGA8* owing to monomorphicity at these sites [14,23].

Evaluating the credibility of significant associations

To estimate the epidemiologic credibility of associations with polymorphisms showing sub-genome-wide significant association with PD ($P>5\times10^{-8}$), we applied two "credibility" measures for each such result. First, we calculated Bayes factors (BF, expressed here as $\log_{10}$-values, "logBF") assuming an average non-null odds ratio of 1.15, as approximation of a typical "complex disease effect size", and a spike and smear prior distribution of effects [25]. Our

Table 2. Genome-wide significant summary meta-analysis results of the PDGene database in populations of Caucasian and Asian decent.

Caucasian ethnicity

Locus	Polymorphism	Location (hg18)	MAF	Allele contrast	N datasets	N samples	OR (95% CI)	*P*-value	I^2 (95% CI)	HuGENet	BF
GBA	N370S	chr1:153451576	0.01	G vs. A	15	44,851	3.51 (2.55–4.83)	1.44×10^{-14}	38 (0–66)	A	6.6
SYT11/RAB25	chr1:154105678	chr1:154105678	0.02	T vs. C	6	17,300	1.73 (1.48–2.02)	2.35×10^{-12}	0 (0–52)	B*	8.2
PARK16	rs947211	chr1:204019288	0.23	A vs. G	12	69,262	0.91 (0.88–0.94)	8.00×10^{-10}	0 (0–66)	A	6.8
STK39	rs2390669	chr2:168800188	0.13	C vs. A	14	35,159	1.19 (1.12–1.25)	1.37×10^{-09}	18 (0–56)	A	4.9*
MCCC1/LAMP3	rs11711441	chr3:184303969	0.14	A vs. G	25	46,502	0.86 (0.82–0.91)	9.20×10^{-10}	18 (0–50)	A	6.8
DGKQ	rs11248060	chr4:954359	0.12	T vs. C	10	57,716	1.21 (1.15–1.27)	3.04×10^{-12}	11 (0–52)	A	9.2
BST1	rs11724635	chr4:15346199	0.43	C vs. A	26	46,586	0.88 (0.84–0.91)	1.87×10^{-10}	43 (10–64)	A	7.5
SNCA	rs356219	chr4:90856624	0.41	G vs. A	31	79,494	1.29 (1.25–1.33)	6.06×10^{-65}	16 (0–46)	A	61.0
ITGA8	rs7077361	chr10:15601549	0.12	C vs. T	11	61,036	0.88 (0.84–0.92)	1.51×10^{-08}	0 (0–55)	A	5.7
LRRK2	rs1491942	chr12:38907075	0.21	G vs. C	21	34,123	1.17 (1.13–1.22)	6.44×10^{-15}	0 (0–38)	A	11.8
CCDC62/HIP1R	rs10847864	chr12:121892551	0.39	T vs. G	23	38,367	1.15 (1.11–1.18)	4.37×10^{-17}	0 (0–35)	A	14.4
MAPT/STH	H1H2	chr17:42131818–41149582	0.20	H2 vs. H1	37	50,389	0.78 (0.75–0.80)	7.97×10^{-52}	0 (0–29)	A	48.1

Asian ethnicity

Locus	Polymorphism	Location (hg18)	MAF	Allele contrast	N datasets	N samples	OR (95% CI)	*P*-value	I^2 (95% CI)	HuGENet	BF
PARK16	rs823156	chr1:204031263	0.17	G vs. A	5	22,870	0.74 (0.68–0.81)	2.09×10^{-12}	0 (0–58)	A	9.2
BST1	rs4538475	chr4:15347035	0.38	G vs. A	3	20,393	0.80 (0.75–0.86)	9.53×10^{-10}	0 (-)	A	6.8
SNCA	rs6532194	chr4:90999925	0.40	T vs. C	5	22,844	1.29 (1.20–1.39)	4.91×10^{-11}	31 (0–74)	A	8.0
LRRK2	rs34778348	chr12:39043595	0.04	A vs. G	13	10,441	2.23 (1.89–2.63)	2.97×10^{-21}	0 (0–53)	B*	15.2

Whenever multiple polymorphisms showed genome-wide significant association in the same locus, only the variant with the smallest *P*-value is listed here. Note that, overall, 103 PDGene meta-analyses results across the 12 loci listed above yield genome-wide significant evidence for association with PD. For a complete list of these as well as the non-genome-wide significant meta-analysis results performed for the datafreeze, see Table S1. MAF = minor allele frequency in cases and controls combined; N = Number, OR = Odds Ratio; CI = confidence interval; I^2 = estimate of percentage of between-study heterogeneity that is beyond chance. BF = Bayes factor. *Note that additional polymorphisms in these loci showing genome-wide significant association with PD yield are graded with "strong epidemiologic credibility" (grade A) according to the HuGENet criteria [26,27], and a Bayes Factor >5 [25], respectively (see Table S1 for more details).

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second assessment was based on the Human Genome Epidemiology Network's (HuGENet) interim criteria for the assessment of cumulative epidemiologic evidence in genetic association studies [26,27]. The results of these analyses are summarized in Table S1.

There was strong epidemiologic support in both assessments for all loci showing genome-wide significant association. This included several additional polymorphisms in these same loci that only showed sub-genome-wide significant association. However, there was no additional sub-genome-wide significantly associated locus that received unequivocally strong support from both credibility assessments (Table S1). In this list, the strongest support was assigned to SNP chr6:32588205 in the *HLA* locus receiving the best possible grade in the HuGENet criteria (grade A), but more moderate support in the Bayesian analyses (logBF = 4.4). However, the relevance of this assessment needs to be evaluated as the underlying analysis was only based on four GWAS datasets.

Discussion

The PDGene database represents a comprehensive, regularly updated and freely available online research synopsis of genetic association studies in PD. Detailed summaries of the most compelling findings are provided within an easy-to-use, dedicated online framework, displaying forest plots, cumulative meta-analyses, and an up-to-date ranking of "Top Results". To allow comparison of PDGene results with association findings from other complex diseases and to facilitate their interpretation with respect to functional genetics data, all meta-analysis results have been ported as a customized track onto the UCSC Genome Browser. This will also allow for a integration and visualization [28] of association results from large-scale resequencing data (e.g. from whole-exome or whole-genome studies) into PDGene once these become available.

To the best of our knowledge, our study represents the most comprehensive research synopsis in the field of PD genetics. In addition, it represents the first disease-specific genetic database that allows a systematic and exhaustive inclusion of GWAS data, and may serve as a model for similar databases in other complex genetic diseases. Owing to our multi-pronged data retrieval and analysis protocol we were able to perform meta-analyses on the vast majority of PD risk-gene candidates, including those "featured" as top association results in all published GWAS. In particular, this includes the five novel loci recently featured in the recent GWAS meta-analysis [21]. Through collaboration with other PD genetics laboratories we obtained independent summary data for these and 142 additional SNPs, substantially extending the hitherto available evidence. Taken together, our analyses provide unequivocal evidence that *BST1*, *CCDC62/HIP1R*, *DGKQ/GAK*, *GBA*, *ITGA8*, *LRRK2*, *MAPT*, *MCCC1/LAMP3*, PARK16, *SNCA*, *STK39*, *SYT11/RAB25* represent genuine PD risk loci, while the role of several other loci (e.g. *ACMSD/*

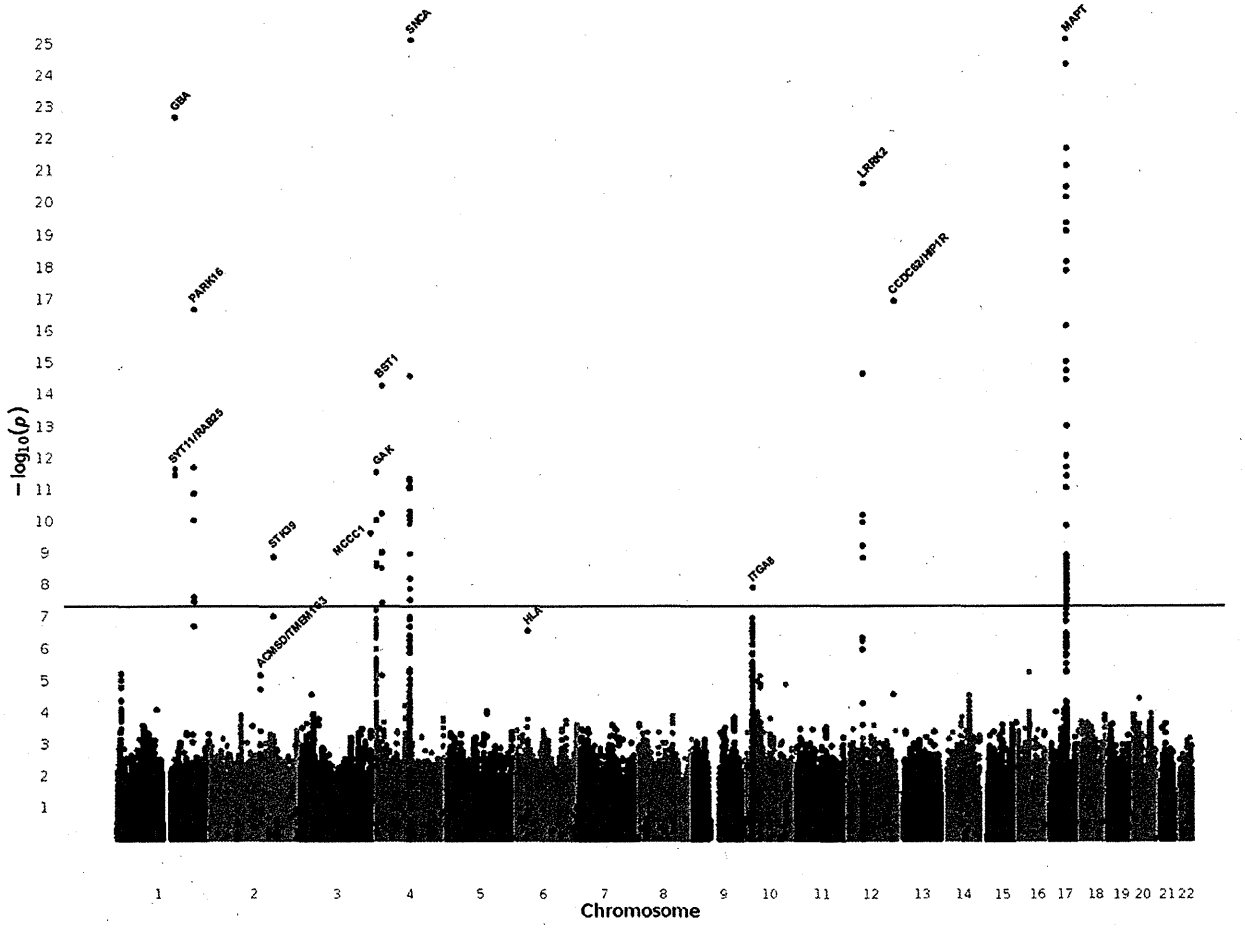

Figure 1. Manhattan plot of all meta-analysis results performed in PDGene. This summary combines association results from 7,123,986 random-effects meta-analyses based on the March 31st 2011 datafreeze of the PDGene database. Results are plotted as $-\log_{10}$ *P*-values (y-axis) against physical chromosomal location (x-axis). Black and grey dots indicate results originating exclusively from the three fully publicly available GWAS datasets [10,12,13] (see Methods), while green dots are based on a combination of smaller scale studies, supplemented by GWAS datasets (where applicable). Gene annotations are provided for genes highlighted in the main text.
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TMEM163, and the *HLA* locus) remains to be determined. The unpublished data aggregated here from various PD genetics groups for selected candidate genes represents the first step towards a systematic meta-analysis across the full GWAS datasets from the same populations. Once completed, the results of this "mega" meta-analysis will be posted on the PDGene database, allowing users to browse the complete results via the customized genome browser track already in place.

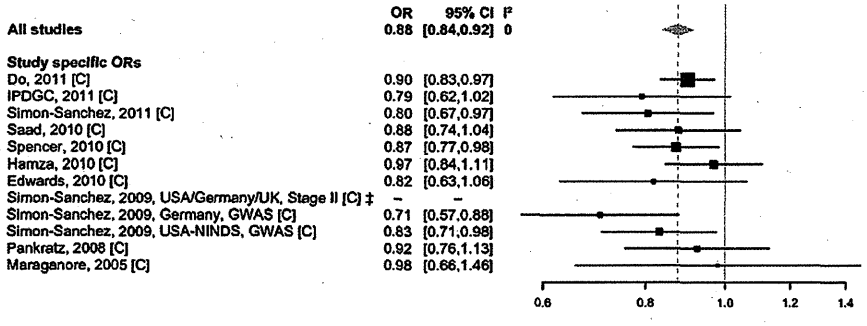

Figure 2. Forest plot of the meta-analysis of rs7077361 in *ITGA8*. Study-specific allelic odds ratios (ORs, black squares) and 95% confidence intervals (CIs, lines) were calculated for each included dataset. The summary OR and CI was calculated using the DerSimonian Laird random-effects model (grey diamond) [31]. C = Caucasian ancestry.
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Of particular interest are loci with unusually large effect sizes. While most loci in PDGene have only small effects on PD risk (with ORs ranging from 1.10 to 1.35, which are typical for complex diseases), for some loci much larger ORs were estimated (i.e. *GBA* [OR 3.51 in Caucasians], *LRRK2* [OR 2.23 in Asians], and *SYT11/RAB25* [OR 1.73 in Caucasians], see Table 2). The risk-allele frequencies at these polymorphisms are typically rather small (i.e. below 0.05), resulting in low population attributable risks for these loci (for the above mentioned loci individually less than 2%).

Interestingly, the meta-analysis results of *GBA* N370S as well as the *LRRK2* rs34778348 are solely based on candidate-gene approaches since these SNPs are not on any of the current GWAS arrays or imputation reference panels. Thus, even in the "GWAS era" smaller-scale, non-GWAS but "focused" genetic studies, will likely continue to play an important role. This is also true when it comes to providing independent replication of proposed disease associations and/or when validating imputation-derived results by direct genotyping in sufficiently sized datasets. PDGene systematically concatenates all these different types of data into one database framework, vastly facilitating an assessment of the overall evidence for any given SNP or locus.

The strength of our approach is further exemplified by the identification of genome-wide significant association between disease risk and a SNP in *ITGA8*, which was not featured as a relevant PD gene in any previous study. *ITGA8* (encoding integrin alpha 8, a type-I transmembrane protein) is functionally interesting as it is expressed in brain [29], mediates cell-cell interactions and regulates neurite outgrowth of sensory and motor neurons [30]. Additional studies are needed to further assess the potential role of this gene in PD pathogenesis. Furthermore, PDGene shows that two additional loci, not highlighted by the recent GWAS meta-analysis [21], yield genome-wide signficiant results in the PDGene meta-analyses, i.e. PARK16, originally implicated as a PD susceptibility locus in an Asian GWAS [14] but not highlighted in the recent GWAS meta-analysis on Caucasian samples [21] and *GBA*, a gene that was found soley by candidate-gene approaches. Another strength of our study is that it combines genetic data from currently more than 50 different countries allowing a systematic assessment of genetic associations across populations of different ethnic descent. For instance, these analyses suggest that variants in *BST1*, *LRRK2*, the PARK16 locus, and *SNCA* show genome-wide significant association with PD risk in both Caucasian and Asian-descent samples. Furthermore, the recently described Caucasian GWAS loci *CCDC62/HIP1R*, *MCC1*, and *STK39* [21] also show similar effect size estimates in populations of Asian-descent [23]. PD association data originating from other ethnic groups are still relatively scarce. However, they could easily be added to the already existing data on the respective polymorphisms available on PDGene.

In summary, we have created a continuously updated online resource for genetic association studies in the field of PD. Synthesizing essentially all available data in the field led to the identification of *ITGA8* as a novel potential PD risk locus. Our quantitative approach to data integration across a multitude of different study designs can be readily scaled to include large-scale resequencing efforts that will emerge over the coming years, making the complex field of PD genetics accessible to a broad range of investigators.

Methods

Note that the following section only provides a brief summary of the methods applied to our study. A much more detailed description can be found in Text S1.

Literature searches

Inclusion criteria. For inclusion in PDGene, a study has to meet three criteria: 1) It must evaluate the association between a bi-allelic genetic polymorphism (minor allele frequency $\geq$0.01 in the healthy control population of at least one study) and Parkinson's disease (PD) risk in datasets comprised of both affected (defined as clinically and/or neuropathologically diagnosed "Parkinson's disease") and unaffected individuals; 2) it must be published in a peer-reviewed journal; 3) it must be published in English. For this manuscript, we also included data on ten SNPs generated in the GEO-PD Consortium datasets [14,23] and obtained data for the newly identified SNP rs7077361 in *ITGA8* from the Japanese GWAS dataset [14].

Exclusion criteria. In brief, genetic association data of the following studies were excluded from the meta-analyses (see Text S1 for details): family-based studies without available subject-level data (however, unrelated case-control data enriched for familial cases were not excluded), studies investigating only disease controls, multi-allelic polymorphisms, and studies of polymorphisms in mitochondrial DNA. We also excluded genetic data of apparently "poor" quality if discrepancies could not be resolved after contacting the study authors (e.g. inadequate genotyping/sequencing protocols or discrepancies in terms of allele names or frequencies when compared with public databases; more details can be found in Text S1).

Search strategies. Our literature searches until March 31st, 2011, yielded 27,210 articles, which were screened for eligibility using the title, abstract, or full-papers, as necessary. Additional screening of bibliographies in reviews, published meta-analyses, and original genetic association studies were also performed. Overall, full text versions of 1,534 articles were obtained. Following the inclusion and exclusion criteria outlined above, 828 articles were included in PDGene until March 31st 2011 (also see Figure 3).

Statistical analyses

Meta-analyses. Random-effects allelic meta-analyses [31] were performed if a minimum of four independent datasets existed per polymorphism. Summary odds ratios [ORs] and 95% confidence intervals [CIs] were calculated irrespective of ethnic descent as well as for distinct ethnic groups (i.e. Caucasians, and Asians) if sufficient data were available. In addition, we performed a number of sensitivity analyses (excluding the initial studies and datasets in which HWE was violated in control individuals), systematically assessed between-study heterogeneity (via I^2), and assessed the credibility of each at least nominally significant meta-analysis result by calculating Bayes factors (BF; here expressed as log10(BF) = "logBF") [25] and by determining a grading score developed by the Human Genome Epidemiology Network (HuGENet) [26,27].

Assessment of small-study bias/publication bias. This is of particular importance in meta-analyses of published association data and was carefully addressed here: First, we added *publicly* available GWAS data [10,12,13] to the vast majority of SNPs. Since these data are typically unbiased, this should decrease the potential for small-study bias/publication bias. Secondly, for 147 SNPs of the core PDGene meta-analyses that showed statistically suggestive results ($P \leq 0.1$), we obtained additional data from all currently published, but *not publicly* available GWAS datasets, further decreasing a potential impact of small-study bias/publication bias. Thirdly, we directly assessed the evidence for small study bias by applying a recently proposed regression test [32] on all nominally significant ($P<0.05$) meta-analysis results. The results of these analyses are fully displayed in Table S1.