ISPD loss-of-function mutations disrupt dystroglycan O-mannosylation and cause Walker-Warburg syndrome

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Walker-Warburg syndrome (WWS) is clinically defined as congenital muscular dystrophy that is accompanied by a variety of brain and eye malformations. It represents the most severe clinical phenotype in a spectrum of diseases associated with abnormal post-translational processing of α-dystroglycan that share a defect in laminin-binding glycan synthesis1. Although mutations in six genes have been identified as causes of WWS, only half of all individuals with the disease can currently be diagnosed on this basis2. A cell fusion complementation assay in fibroblasts from undiagnosed individuals with WWS was used to identify five new complementation groups. Further evaluation of one group by linkage analysis and targeted sequencing identified recessive mutations in the ISPD gene (encoding isoprenoid synthase domain containing). The pathogenicity of the identified ISPD mutations was shown by complementation of fibroblasts with wild-type ISPD. Finally, we show that recessive mutations in ISPD abolish the initial step in laminin-binding glycan synthesis by disrupting dystroglycan O-mannosylation. This establishes a new mechanism for WWS pathophysiology.

The hallmark of dystroglycanopathies—and the common pathogenic denominator in all individuals with WWS (MIM 236670)—is loss of functional glycosylation of α-dystroglycan3. Lack of proper α-dystroglycan glycosylation reduces binding to extracellular matrix proteins, as ligand binding is mediated through the sugar moiety on α-dystroglycan4. All six of the genes in which causative mutations in WWS have previously been found code for known or putative glycosyltransferases; mutations in these genes show autosomal recessive inheritance patterns and result in abnormal α-dystroglycan glycosylation5. Nevertheless, approximately half of the population with WWS has no mutation in these known genes6, emphasizing the need for discovery of other causative genes that are disrupted in WWS.

We established a complementation assay to enable us to identify additional genes that contribute to WWS pathology. This assay was developed based on a panel of skin fibroblasts derived from six individuals with genetically defined but heterogeneous dystroglycanopathy (Supplementary Table 1), in which protein blotting revealed a lack of functional glycosylation (defined as immunoreactivity to IIH6, a monoclonal antibody specific for the requisite sugar moiety) and an inability to bind laminin (Fig. 1a). These pathological characteristics are consistent with previously published data obtained from various dystroglycanopathy tissues3,6 and cells7–9. Notably, the degree of α-dystroglycan hypoglycosylation varied with the gene mutated, and the differing molecular weights of α-dystroglycan produced in the individual fibroblast populations are hypothesized to reflect abnormalities at different steps in α-dystroglycan biosynthesis (Fig. 1a). In fibroblasts from each population of affected individuals, the α-dystroglycan glycosylation defect was rescued by introducing a wild-type copy of the mutant gene. For example, in fibroblasts from individuals with WWS with known POMT1 mutations, α-dystroglycan functional glycosylation was restored by adenovirus-mediated gene transfer of POMT1 but not by expression of the other known WWS-associated genes (Fig. 1b). This complementation assay was adapted to On-Cell protein blotting technology, and rescue of α-dystroglycan functional glycosylation was shown for all known WWS-associated genes (Fig. 1c).

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Previously, it was shown that forced overexpression of the glycosyltransferase LARGE induces α-dystroglycan hyperglycosylation in control cells and bypasses the α-dystroglycan glycosylation defect in cells from individuals with dystroglycanopathy. However, we now show that the ability of LARGE to hyperglycosylate α-dystroglycan is dependent on the availability of O-mannosyl phosphate acceptor sites and correlates with the severity of the clinical phenotype (Fig. 1d).

We next applied the On-Cell complementation assay to fibroblasts derived from a cohort of 63 individuals with dystroglycanopathy (Supplementary Fig. 1), identifying 11 subjects with WWS in 10 unrelated families who we postulated to have mutations in genes not previously implicated in WWS. Our first step toward defining the genetic basis for WWS in these individuals was to establish complementation groups; to this end, we adapted a cell fusion approach that is commonly used in yeast and that has also proven successful in mammalian cells. Whereas fusion is achieved by mating in the case of yeast cells, polyethylene glycol (PEG) treatment is used to induce fusion in mammalian cells. We hypothesized that fusion between co-cultured cells from affected individuals harboring recessive mutations in the same gene would not rescue the α-dystroglycan glycosylation defect, whereas fusion between cells from affected individuals with independent genetic defects would result in successful rescue. Immunofluorescence analysis identified IIH6-positive fused cells (as indicated by the presence of multiple nuclei) only when two cell lines from genetically different individuals were co-cultured; for example, complementation between POMT1- and FKTN-deficient fibroblasts from subjects with WWS is shown (Fig. 2a). Two-way fusions of WWS-derived cells with mutations in each of the known genes rescued α-dystroglycan glycosylation (data not shown). Application of the
PEG fusion approach to all 11 subjects with WWS for whom a genetic cause had not been determined led to the identification of five separate complementation groups (Fig. 2b,c), suggesting that five new causative genes in WWS were represented in this small cohort. Four complementation groups were represented by a single subject with WWS, and one complementation group consisted of seven subjects with WWS. If mutations in a single newly implicated gene are responsible for disease in all seven subjects, then the genetic alterations in this complementation group likely represent a relatively common cause of WWS.

All seven subjects within this complementation group met the classic diagnostic criteria for WWS (Supplementary Table 2). Two of the subjects, P5 (refs. 6,14) and P6 (ref. 15), were described as having WWS in previously published studies. In the case of P1, brain magnetic resonance imaging (MRI) performed at 3 days and at 5 months of age showed hydrocephalus, cobblestone lissencephaly of the cerebral cortex, severe brainstem hypoplasia with a kink at the isthmus and severe hypoplasia of the cerebellum (Fig. 3a). This subject also showed evidence of severe muscular dystrophy (Fig. 3b), bilateral microphthalmia with cataracts and arrested retinal development. Immunofluorescence and protein blot analysis of a skeletal muscle biopsy (Fig. 3b), showing complete loss of functional glycosylation and receptor function (Fig. 3b,c). Comparative analysis of the status of α-dystroglycan glycosylation in fibroblasts from five different subjects within this same WWS complementation group confirmed that all shared a defect in α-dystroglycan processing, showing complete loss of functional glycosylation and laminin binding (Supplementary Fig. 2). Moreover, the loss of post-translational modification of α-dystroglycan and its consequent shift to a lower molecular weight were comparable in all samples, consistent with the hypothesis that individuals from this complementation group share a common genetic defect.

Our first step toward identifying the underlying genetic defect in the large complementation cohort was to perform linkage analysis. As reliable family history regarding consanguinity was not available for all subjects, regions of homozygosity by descent were identified using high-resolution SNP arrays. Besides the sibling pair of subjects P2 and P3, four of the five unrelated subjects showed multiple long (>10 cM) stretches of homozygosity, suggesting some degree of consanguinity (Supplementary Fig. 3). We searched for chromosomal regions where P2 and P3 were identical on both alleles and where all or a subset of the four suspected consanguineous subjects were homozygous. All 7,113 coding exons across the 14 identified overlapping intervals were subjected to targeted sequencing (Supplementary Table 3). All seven samples were barcoded, pooled, captured by a custom-designed capture array and sequenced on a lane of an Illumina HiSeq2000 flow cell as

Table 1 Summary of pathogenic ISPD mutations detected in this study

| Subject | Zygosity | Chr. | Genomic position (Build 37) | Nucleotide variant | Amino-acid alteration |
|---------|----------|------|-----------------------------|-------------------|----------------------|
| P1      | Heterozygous | 7 | 16415758 | c.643C>T | p.Gln215* Non-sense mutation |
| P1      | Heterozygous | 7 | g.(16107358–16115680), (16289931–16297326)del |                  | Deletion of exons 9 and 10 |
| P2 and P3 (siblings) | Heterozygous | 7 | 16348146 | c.789+2T>G | Splicing defect |
| P4      | Heterozygous | 7 | 16445940 | c.277_279del ATT | p.Ile93del Single amino-acid deletion |
| P5      | Homozygous | 7 | 16131322 | c.1354T>A | p.*452Arg Mutation of original stop codon, next stop codon 27 aa downstream |
| P6      | Homozygous | 7 | 16255823 | c.1120–1G>T | Splicing defect |
| P7      | Homozygous | 7 | g.(16401191–16406273), (16409318–16431594)del | c.550C>T | p.Arg184* Non-sense mutation |

Chr. chromosome.
a 50-bp paired-end run. Sequence data were processed in a custom-built analysis pipeline, and, after variant filtering was applied, six genes were identified in which at least two independent protein-damaging variants passed hard-filtration criteria (Supplementary Table 4). On the basis of genetic evidence, ISPD was determined to be the most likely candidate gene. After manually examining variants that did not meet filtration criteria and augmenting the data set with Sanger sequencing results for P5, for whom there was poor coverage, a total of four heterozygous and four homozygous variants were found in ISPD that occurred as multiple rare variants in all six independent affected individuals (Table 1 and Supplementary Fig. 4). All mutations were predicted to damage or abolish protein function, as expected in individuals with a severe form of dystroglycanopathy, such as WWS. In addition, ISPD is localized to chromosome 7p21.2, a region in which three of the four suspected consanguineous subjects had intervals of homozygosity longer than 10 cM and where P2 and

**Figure 4** Identification and validation of ISPD as a disease-associated gene in subjects with WWS. (a) Alignment of identity-by-descent (IBD) and homozygosity-by-descent (HBD) intervals among ISPD-deficient subjects on chromosome 7 is shown. Top, genomic position in hg19 coordinates; bottom, chromosome bands. The minimal region of overlap where three of the four samples from suspected consanguineous individuals were homozygous and where P2 and P3 share both parental alleles is highlighted by a red box. (b) Genes within the overlap region in a are shown in a magnified view. (c) Schematic (not to scale) of the ISPD exon-intron gene structure. Human ISPD cDNA (5,524 bp; NM_001101426) contains 10 coding exons spread across 333,796 bp of genomic DNA. All identified pathogenic ISPD protein changes are indicated, as are regions of exon deletion and splice-site mutations. Coding exons, black boxes; UTRs, open boxes. (d) On-Cell protein blot–based complementation assay of fibroblasts from a control and ISPD-deficient subject with WWS after nucleofection with a wild-type or mutant ISPD expression construct. Rescue of α-dystroglycan functional glycosylation was detected with antibody to glycosylated α-dystroglycan (glyco α-DG). (e) Adenovirus-mediated ISPD gene transfer rescues the α-dystroglycan glycosylation defect in cells from ISPD-deficient subject P2. WGA-enriched cell lysates from fibroblasts were subjected to immunoblotting with antibodies to glycosylated α-dystroglycan (glyco α-DG), core α-dystroglycan (core α-DG), Myc and β-dystroglycan (β-DG) and to laminin overlay. Infection with ISPD-Myc adenovirus restored functional glycosylation in ISPD-deficient cells from P2 but did not substantially alter α-dystroglycan functional glycosylation in control cells.
to use an alternative, ISPD-independent mevalonate (MVA) pathway for isoprenoid synthesis\(^{20}\). As the MEP pathway involving ISPD is postulated to be absent from animals, the specific role of ISPD in humans, especially in regard to α-dystroglycan glycosylation, is unclear.

To investigate the role of ISPD in α-dystroglycan glycosylation, we tested subjects with WWS who had mutations in ISPD for changes in any known step in laminin-binding glycan synthesis. Notably, protein O-mannosylation, the initiating step of this pathway, was markedly reduced in fibroblasts lacking functional ISPD, as were downstream events like O-mannosyl phosphorylation and LARGE-induced hyperglycosylation (Fig. 5a–c). These findings suggest that ISPD function is crucial for efficient POMT-dependent O-mannosylation and subsequent glycosylation of α-dystroglycan (Supplementary Fig. 8).

In this study, we have identified a new disease-associated gene in individuals with WWS by using a fibroblast complementation assay in combination with targeted sequencing. This approach provided conclusive genetic and biochemical evidence that recessive mutations in ISPD lead to impaired α-dystroglycan O-mannosylation, establishing a new pathway and mechanism for disease in WWS. Further studies are needed to determine how mutations in ISPD influence protein O-mannosylation, as this is the first WWS-associated gene without proposed glycosyltransferase activity and a direct role in α-dystroglycan glycosylation.

**METHODS**

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

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**AUTHOR CONTRIBUTIONS**

T.W., H.L., S.F.N. and K.P.C. designed the research. T.W. performed the research and analyzed the data. H.L. and S.F.N. performed SNP analysis, next-generation
sequencing and data filtering. M.L. and S.S. carried out POMT enzyme activity assays. T.Y.-M. performed α-dystroglycan orthophosphate cell labeling experiments. D.B.V.d.B. performed qRT-PCR expression analysis. D.V. carried out antibody affinity purification and labeling. T.L.W. carried out Sanger sequencing of known WWS-associated genes. S.A.M. performed muscle histology and clinical data interpretation. H.S., J.V., S.C., F.M., T.V., A.S.L., W.B.D. and K.D.M. provided clinical data and/or fibroblast samples from individuals with WWS. K.P.C. supervised and mentored the project. T.W. and K.P.C. wrote the initial manuscript, and all authors approved and commented on the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Subjects and samples. We obtained and tested all tissues and cells from affected persons in agreement with the guidelines set out by the Human Subjects Institutional Review Board of the University of Iowa; informed consent was obtained from all subjects or their legal guardians. More detailed information on fibroblasts from controls and affected individuals with dystroglycanopathy with known genetic defects is summarized in Supplementary Table 1.

Cell culture. Cells were maintained at 37 °C and 5% CO2 in DMEM medium supplemented with 20% FBS and 0.5% penicillin-streptomycin (Invitrogen).

Biochemical analyses. The monoclonal antibodies to the fully glycosylated form of α-dystroglycan (IIH6 and VIA4) and to β-dystroglycan (AP8) have been characterized previously. GT20 antibody (to core α-dystroglycan) from goat antisera was raised against the dystrophin-glycoprotein complex (DGC) in its entirety and purified against a hypoglycosylated full-length α-dystroglycan–human IgG Fc fusion protein. Additionally, antibodies against the protein backbone of α-dystroglycan were generated by injecting a keyhole limpet hemocyanin (KLH)-conjugated synthetic peptide consisting of amino acids 485–514 of human dystroglycan (Swiss-Prot P14118) into rabbits (G6317, Genemed Biosynthesis). The antibody to core α-dystroglycan was affinity purified using BSA-conjugated C-terminal peptide. Mouse monoclonal antibody to Myc (4A6) was purchased from Millipore.

Assay for protein O-mannosyltransferase activity. Protein O-mannosyltransferase activity was determined on the basis of the amount of [3H]-mannose transferred from Dol-P-[3H]-mannose to an α-dystroglycan glutathione-S-transferase fusion as described elsewhere.

[32P] Orthophosphate labeling of cells. Phosphorylation of α-dystroglycan in fibroblasts from affected individuals was determined on the basis of the incorporation of [32P] into a secreted Fc-tagged α-dystroglycan recombinant (DGECs), as described elsewhere.

Adenovirus generation and gene transfer. E1-deficient recombinant adenoviruses (Ad5CMV-DAG1, Ad5CMV-DGFC5, Ad5CMV-POMTI/RSVeGFP, Ad5CMV-POMTI2/RSVeGFP, Ad5CMV-POMGnt1/RSVeGFP, Ad5CMV-FKTV/RSVeGFP, Ad5CMV-FKTK/RSVeGFP and Ad5CMV-LARGE/RSVeGFP) were generated by the University of Iowa Gene Transfer Vector Core and described previously. Similarly, Ad5CMV-ISPD-Myc/RSVeGFP was generated by cloning the ORF corresponding to human ISPD (NM_00110426) in frame with a sequence encoding a C-terminal Myc tag into the polynucleotide region of Ad5CMV-NP-A. Cells were infected with viral vector for 12 h at an MOI of 400. We examined cultures 3–5 d after treatment. We also used nucleofection as a non-viral method for gene transfer into cells. Nucleofection of fibroblasts was performed using the Human Dermal Fibroblast Nucleofector Kit, according to an optimized protocol provided by the manufacturer (Amaxa Biosystems).

ISPD expression vector construction. cDNA was generated from fibroblasts from a control individual and from subject P6 with ISPD-deficient WWS (in-frame deletion of exon 3, 30 aa deletion) fibroblasts. The C-terminally Myc-tagged coding regions of human wild-type ISPD (1,395 bp) and mutant ISPD (1,245 bp) were amplified from cDNA by PCR, using primer pair 7717/7718 (Supplementary Table 5). The amplified PCR product was subcloned using the pcDNA3.1/V5-His TOPO TA Expression Kit (Invitrogen).

Immunohistochemical analysis. Cryosections of skeletal muscle biopsies (10 μm thick) were processed for immunofluorescence as described. Mouse monoclonal antibody to laminin (2E3/anti-merosin, clone SH2) was purchased from Millipore. Cultured cells were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min, before being blocked and incubated with primary antibody. Slides were visualized on an Axio Imager.M1 microscope (Zeiss).

Glycoprotein enrichment and biochemical analysis. WGA-enriched glycoproteins of frozen samples and cultured cells were processed as described. Immunoblots were performed on PVDF membranes as described. Blots were developed with infrared (IR) dye–conjugated secondary antibodies (Pierce) and scanned with an Odyssey infrared imaging system (LI-COR Bioscience). Laminin overlay assays were performed as previously described.

On-Cell complementation assay of fibroblasts from subjects with WWS. Cells (2 × 104) were seeded into a 48-well dish. The next day, cells were coinfectd with Ad5CMV-DAGI at an MOI of 200 for signal enhancement and Ad5 complementation constructs at an MOI of 200 (except for Ad5CMV-FKTR/RSVeGFP, which was used at an MOI of 40) in growth medium. After 4 d, the cells were washed in TBS and fixed with 4% paraformaldehyde in TBS for 10 min. After blocking with 3% dry milk in TBS with 0.1% Tween (TBS-T), the cells were incubated with primary antibody (to glycosylated α-dystroglycan, IIH6) in blocking buffer overnight. For developing the On-Cell protein blots, we conjugated goat antibody to mouse IgM (Millipore) with IR800CW dye (LI-COR), subjected the sample to gel filtration and isolated the labeled antibody fraction. After staining with IR800CW-labeled secondary antibody in blocking buffer, cells were washed in TBS, and we scanned the 48-well plate with an Odyssey infrared imaging system. For cell normalization, DRAQ5 cell DNA dye (Biostatus Limited) was added during incubation with the secondary antibody.

PEG-induced fusion of dermal fibroblasts. Cell fusion assays were performed as published previously. In brief, cells were seeded into a 48-well dish or onto a tissue culture chamber slide. When the cells reached 90–100% confluency, the culture medium was removed, and cell fusion was induced by adding 55% PEG1500 in DMEM to the cell monolayer and incubating for 1 min at room temperature. The fusion solution was then discarded, and the cell monolayer was rapidly rinsed four times with complete growth medium at room temperature and then returned to the 37 °C tissue culture incubator. The next day, fusion-induced cells were infected with Ad5CMV-DAGI at an MOI of 200 for signal enhancement. After 4 d, cells were fixed and stained.

Linkage analysis. Genomic DNA samples were genotyped on an Illumina Omni-1 Quad BeadChip. Identity-by-descent (IBD) and homozgosity-by-descent (HBD) analyses were performed using a custom Mathematica script (Wolfram Research; B. Merriman, available on request), which compared genomes for genotype identity and identified long intervals of homozgosity.

Targeted next-generation sequencing. Regions over 10 Mb in length where the sibling pair of P2 and P3 showed IBD for both alleles were selected, as were regions that were homozygous for over 2 cM in one or more of the four samples suspected to come from consanguineous individuals (P4, P5, P6 and P7), and were used to create a custom capture array. Using Agilent eArray, a 244K comparative genomic hybridization (CGH) array was designed to target in total 7,113 coding exons across 14 overlapping intervals (Supplementary Table 3). Repeat regions were excluded. Genomic DNA (3 μg) was used to generate each sequencing library, using the Agilent SureSelect Target Enrichment System for Illumina Paired-End Sequencing Library Protocol (version 2.0.1); the only difference was that seven different custom-made, barcode-adapters were used in place of the commercially available adapter (sequences available on request). After amplification, samples were pooled at equal molar concentration, captured on one array following an in-house protocol and sequenced on the Illumina HiSeq2000 as 50-bp paired-end reads.

Sequence read alignment. Barcodes were removed from sequence reads, and sequences were aligned to the Human reference genome Build 37 (hg19) using Novoalign and Novosalign from the Novocraft Short Read Alignment Package (see URLs). Data were processed with SAMtools (version 0.1.15, see URLs), and potential PCR duplicates were removed using Picard (see URLs). The mean coverage achieved within the intended captured region was 35.9x per base, with 82.2% of the targeted base pairs covered at ≥10×. Local realignment was performed using the GATK32,33 and the glnm tool.

Variant calling. Variants were called using the GATK Unified Genotyper tool simultaneously for all seven samples. Small indels were called with the -glm
DINDEL option. Variants with a phred-scaled Q score of ≥50.0 were reported as PASSed calls, and those with a Q score of ≥10.0 and <50.0 were reported as Low Qual calls. Using the GATK VariantFiltrationWalk tool, both SNPs and indels were hard filtered to eliminate low-quality variants. The following filtering parameters were used, which were suggested to be standard by GATK, to remove likely false positive variants: (i) clusterWindowSize of 10, (ii) mapping quality of zero (MAPQ0) of >40, (iii) quality-by-depth (QD) of <5.0 and (iv) strand bias (SB) >−0.10. The GATK VariantEval tool was used to collect the statistics for the PASSed variants (Supplementary Table 6a).

**Variant annotation.** PASSed variants that were not found in dbSNP132 were annotated using SeattleSeq Annotation version 6.16 (see URLs); single-nucleotide variants (SNVs) and indels were annotated separately. Variants present in the 1000 Genomes Project database (March 2010 release) or dbSNP131, as well as those that resulted in coding synonymous changes or affected sequences outside of a coding region, were removed from further analysis (Supplementary Table 6b). The remaining 84 variants were prioritized on the basis of the frequency of coexistence within the same gene.

**Copy-number variant (CNV) detection.** To identify exonic deletions within the ISPD gene, the log R ratio (LRR) of the coverage (Cov), Cov_{i,j}/Cov_{i,P4} was calculated for each exon i in each sample j using ExomeCNV with subject P4 as a control.21

**Sanger sequencing.** Genomic DNA was extracted from dermal fibroblasts or transformed lymphoblastoid cell lines using standard methods (Qiagen DNeasy Blood & Tissue Kit). Coding regions (ten exons) and exon-intron boundaries of ISPD were amplified using PCR (primers sequences and PCR conditions are available upon request). Primer sets for PCR were designed using the web-based design tool ExonPrimer. After PCR amplification, purified products were evaluated by Sanger sequencing using standard protocols.

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