A POGLUT1 mutation causes a muscular dystrophy with reduced Notch signaling and satellite cell loss

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Abstract

Skeletal muscle regeneration by muscle satellite cells is a physiologic mechanism activated upon muscle damage and regulated by Notch signaling. In a family with autosomal recessive limb-girdle muscular dystrophy, we identified a missense mutation in POGLUT1 (protein O-glucosyltransferase 1), an enzyme involved in Notch posttranslational modification and function. In vitro and in vivo experiments demonstrated that the mutation reduces O-glucosyltransferase activity on Notch and impairs muscle development. Muscles from patients revealed decreased Notch signaling, dramatic reduction in satellite cell pool and a muscle-specific α-dystroglycan hypoglycosylation not present in patients’ fibroblasts. Primary myoblasts from patients showed slow proliferation, facilitated differentiation, and a decreased pool of quiescent PAX7+ cells. A robust rescue of the myogenesis was demonstrated by increasing Notch signaling. None of these alterations were found in muscles from secondary dystroglycanopathy patients. These data suggest that a key pathomechanism for this novel form of muscular dystrophy is Notch-dependent loss of satellite cells.

Keywords muscular dystrophy; Notch; O-glycosylation; POGLUT1; satellite cell

Introduction

Cell surface glycans are diverse in structure and function and play critical roles in many biological processes including infection, cancer, and development (2009). Broadly speaking, glycans affect the function of proteins to which they are linked by modulating their structure and/or by serving as a direct recognition signal for other proteins (Varki & Sharon, 2009). Glycans are involved in protein folding, stability, and trafficking and regulate the activity of important signaling pathways such as Notch, WNT, BMP, and Hedgehog (Christian, 2000; Yan & Lin, 2009; Jafar-Nejad et al, 2010; Takeuchi & Haltiwanger, 2014). Not surprisingly, aberrant glycosylation leads to a variety of human diseases, the list of which is growing (Freeze et al, 2014).

Protein glycosylation can directly regulate signaling events. A clear example of this is the regulation of the evolutionarily conserved Notch signaling pathway, which is an intercellular...
communication mechanism widely used during animal development (Hori et al., 2013). The extracellular domain of the Notch receptor is modified with O-fucose and O-glucose glycans (Moloney et al., 2000). The structures of these glycans change in a tissue-specific and developmentally regulated manner (Johnston et al., 1997), and altering Notch glycosylation dramatically affects its activity (Stanley & Okajima, 2010; Takeuchi & Haltiwanger, 2010). One of the cell types whose regulation by Notch signaling has been intensely studied in recent years is the muscle-specific adult stem cell called the satellite cell (SC) (Mourikis & Tajbaksh, 2014). SCs reside between the basal lamina and sarcolemma of myofibers (Mauro, 1961) and are the primary contributors to skeletal muscle growth and repair (Collins et al., 2005). Despite continuous regeneration, pools of SC are maintained in healthy muscle. This is accomplished through asymmetric cell divisions of SC, which give rise to both self-renewing SC and to committed myogenic progenitors that differentiate (Kuang et al., 2007; Sacco et al., 2008). In this process, the Notch pathway is key for maintaining quiescence in SC and for homing of SC-derived myoblasts (Bjornson et al., 2012; Brohl et al., 2012). A recent report indicates that mice in which Notch signaling is specifically blocked in SCs exhibit a decrease in the number of SCs and histological features of muscular dystrophy even upon normal daily activity (Lin et al., 2013), suggesting that Notch-mediated maintenance of an active SC pool is essential for repairing muscle damage caused by regular activity and for maintaining healthy muscle. Muscle dystrophies are inherited disorders characterized by progressive weakness due to skeletal muscle degeneration, due to mutations in a growing list of responsible genes (Chandrasekharan & Martin, 2010; Rahimov & Kunkel, 2013). However, to date, no primary molecular defects in SC or Notch pathway components have been identified in human muscular dystrophies.

Mutations in several genes disrupt various aspects of the dystrophin-associated glycoprotein complex, which links the cytoskeleton to the extracellular matrix in muscle (Chandrasekharan & Martin, 2010). A key component of this complex is dystroglycan (Ervasti et al., 1990), a transmembrane protein essential for normal basement membrane development and muscle maintenance. Dystroglycan comprises a transmembrane β-subunit non-covalently linked to an extracellular α-subunit containing a mucin-like domain, which is modified with numerous O-linked glycans (Barresi & Campbell, 2006). The β-subunit is linked to the actin cytoskeleton, and the O-linked glycans on the α-subunit are critical for its binding capacity to extracellular matrix proteins such as laminin and agrin (Ervasti & Campbell, 1993; Michele et al., 2002). Mutations in dystroglycan itself result in the primary dystroglycanopathies (Henry & Campbell, 1998; Cote et al., 1999; Hara et al., 2011; Willer et al., 2014; Riemersma et al., 2015; Kanagawa et al., 2016). Secondary dystroglycanopathies are caused by interruption of α-dystroglycan–ligand interactions due to mutations in a growing list of genes [currently eighteen (Bonnemann et al., 2014)] encoding glycosyltransferases and accessory proteins responsible for α-dystroglycan’s extensive posttranslational modifications (Muntoni et al., 2011; Inamori et al., 2012; Yoshida-Moriguchi et al., 2013). These include protein O-mannosyltransferase 1 (POMT1) and POMT2, which add O-linked mannose to α-dystroglycan (Manya et al., 2004; Willer et al., 2004), and like-acetylglucosaminyltransferase (LARGE), which is responsible for the addition of repeating xylose-glucuronic acid units on the O-mannosyl glycans on α-dystroglycan (Inamori et al., 2012). The dystroglycan gene (DAG1) is expressed in SCs (Cohn et al., 2002), but glycosylated α-dystroglycan is not required by myogenic cells during proliferation (Awano et al., 2015), a notion supported by the observation that trace amounts of glycosylated α-dystroglycan are found in C2C12 and human primary myoblasts during proliferation. Cultures of freshly isolated muscle fibers suggest that glycosylation of α-dystroglycan is needed for proliferation of SCs in vivo, but it is not necessary when SCs are removed from their niche (Ross et al., 2012). The transition to larger glycans occurs rapidly after differentiation is induced, and a direct correlation exists between LARGE-dependent extension of O-glycans on α-dystroglycan and its function as an extracellular matrix receptor (Goddeers et al., 2013). Despite extensive studies regarding the genetics of dystroglycanopathies, the responsible gene is still unknown in a significant number of patients; thus, mutations in additional genes will likely be implicated in this group of diseases.

Here, we report a homozygous missense mutation (D233E) in the protein O-glucosyltransferase 1 gene, POGLUT1, in four siblings with autosomal recessive limb-girdle muscular dystrophy. POGLUT1 is the sole enzyme that directly adds O-glucose to a distinct serine residue of epidermal growth factor-like (EGF) repeats containing a CX5X(P/A)C consensus sequence (Rana et al., 2011), many of which are found in Notch extracellular domains (Fernandez-Valdivia et al., 2011; Rana et al., 2011). The D233E mutation dramatically reduces the O-glucosyltransferase activity of POGLUT1, leading to impaired Notch signaling and a dramatic decrease in the number of SCs in adult muscles. Transgenic experiments in Drosophila indicate that D233E impairs the ability of human POGLUT1 to rescue the muscle phenotype caused by the loss of fly Poglut1 activity. Patient muscles show α-dystroglycan hypoglycosylation and decreased binding to laminin, but normal binding to agrin and normal basement membrane structure. Moreover, unlike other dystroglycanopathies, patient fibroblasts exhibit normal α-dystroglycan glycosylation and laminin binding. Together, our findings indicate that exhaustion of the SC pool plays a primary role in this novel form of muscular dystrophy.

Results

Clinical and radiological findings

A consanguineous family from southern Spain comprises 17 individuals spanning three generations (Fig 1A). Four out of five siblings from generation II presented a phenotype consistent with a limb-girdle muscular dystrophy. Specifically, the patients exhibited muscle weakness predominantly in the proximal lower limbs, with onset during the third decade. The disease course was progressive, leading to scapular winging and wheelchair confinement. For more extended clinical data regarding this family, see the Appendix Information, Appendix Fig S1, and Appendix Tables S1 and S2. Serum creatine kinase level was normal in three patients and mildly elevated in one (Appendix Table S1). Muscle biopsies from all four affected siblings revealed histological features ranging from very mild myopathic changes to classic dystrophic pathology (Fig 1A). Proteins typically affected in myopathies displayed normal expression in muscle, except for a reduction in α-dystroglycan
Muscle magnetic resonance imaging (MRI) of the legs revealed a striking pattern of muscle involvement (Fig 1C), with early fatty replacement of internal regions of thigh muscles that spared external areas. This “from inside-to-outside” mode of fatty degeneration progressed over the years and did not match the distribution patterns typically associated with other forms of muscular dystrophies (Appendix Information and Appendix Figs S3 and S4).

Expression and functional modification of α-dystroglycan in patients

Given the key role played by aberrant α-dystroglycan glycosylation and function in a subset of muscular dystrophies and because of the observed decrease in α-dystroglycan levels in patient muscles, we examined the glycosylation status and ligand-binding ability of α-dystroglycan in our patients. Immunofluorescence staining of frozen cross sections from skeletal muscle biopsy with an antibody against glycosylated α-dystroglycan [IIH6 (Ervasti & Campbell, 1991)] revealed a variable reduction in the glycosylated form of α-dystroglycan at the sarcolemma in patients, while antibodies against α-dystroglycan core protein, β-dystroglycan, and laminin α2 showed normal staining (Fig 2A and Appendix Fig S5A). In agreement with this observation, Western blots showed a reduction in α-dystroglycan glycosylation in patient muscle, accompanied by a mild decrease in the molecular weight of glycosylated α-dystroglycan compared with controls. To examine whether decreased α-dystroglycan glycosylation affected binding to ligands, we performed a ligand overlay assay. As shown in Fig 2B, the laminin-binding activity was diminished in muscle. However, the agrin-binding activity to the patients’ muscle extracts showed no difference compared with controls (Fig 2B). Moreover, in skin fibroblasts from patients, the level of both functional α-dystroglycan glycosylation, examined by Western blot and flow cytometry (Stevens et al, 2013b), and its ability to bind laminin (Fig 2B and Appendix Fig S5B) were normal, unlike known secondary dystroglycanopathies, which usually result in decreased functional α-dystroglycan glycosylation in both muscle and the skin fibroblasts (Willer et al, 2012; Carss et al, 2013; Stevens et al, 2013a). Finally, although basement membrane defects are commonly observed in dystroglycanopathies (Yamamoto et al, 1997; Goddeers et al, 2013), transmission electron microscopy showed normal muscle ultrastructure in patients, with no alterations in basement membrane compaction (Fig 2C and Appendix Fig S5C).
These observations suggest that although a partial decrease in α-dystroglycan functional glycosylation is detected in muscle from our patients, decreased α-dystroglycan glycosylation is unlikely to be caused by the same pathomechanism seen in secondary dystroglycanopathies and that a mutation in a gene other than the enzymes directly involved in α-dystroglycan glycosylation might underlie this disease.

D233E mutation in POGLUT1

Homozygosity mapping in DNA samples through genomewide genotyping arrays containing ~ one million single nucleotide polymorphisms (SNPs) revealed a unique (> 500 kb) region of homozygosity identical by descent in the four affected siblings and absent in their
healthy sister (Fig 3A). This 14.6 megabase region on chromosome 3q13.13-q21.2, defined by SNP markers rs13081374 and rs2333038, included 112 protein-coding genes (Fig 3B and C). No structural genomic variations were shared by affected family members, thus excluding gross gene dosage change as a cause of disease. We next performed whole exome sequencing of patients II.4, II.5, and their healthy sister II.3 and found a c.699T>G transition within POGLUT1 (Fig 3D). The polymorphism produces an aspartic-to-glutamic substitution at amino acid residue 233 (p.D233E), which passed all possible filtering, segregated with the disease, and consequently was suspected to be the causal genetic alteration.

Significantly, POGLUT1 is located within the 3q13.13-q21.2 region of homozygosity in the affected siblings (Fig 3C). The mutation was not found in the Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org) [accessed in February 2015], thus discarding this genetic variant in 60,698 individuals (36,673 Europeans). In addition, the D233E mutation was further excluded in 919 DNA samples from 57 populations worldwide (Appendix Table S3).

The mutated aspartic acid residue is located in the capsule-associated protein (CAP)10 domain, 14 amino acids apart from the putative ERD catalytic motif, and is highly conserved across vertebrate species (Fig 3E).

**Expression of POGLUT1**<sub>D233E</sub> **in patients**

We assessed the effect of the D233E mutation on the expression of POGLUT1 in samples from patients. In skeletal muscle, Western blot showed normal protein expression and qRT–PCR assays showed no difference in the POGLUT1 mRNA level compared with controls. In skin biopsy-derived fibroblasts, immunostaining revealed a colocalization of POGLUT1<sup>D233E</sup> with the endoplasmic reticulum, displaying the same pattern as the wild-type POGLUT1 in human control fibroblasts (Appendix Fig S6). These data indicate that the D233E mutation does not affect the expression level or subcellular localization of POGLUT1.

**Biochemical assays of D233E mutation in POGLUT1**

We next analyzed the effect of the D233E mutation on the O-glucosyltransferase activity of POGLUT1 by biochemical assays. Wild-type and D233E mutant forms of human-POGLUT1 were transiently expressed in HEK293T cells and purified from the culture media (Appendix Fig S7A). We examined the enzymatic activity of purified proteins toward a known substrate: bacterially expressed epidermal growth factor-like (EGF) repeat from human coagulation factor IX (hFIX). POGLUT1<sup>D233E</sup> mutant showed significantly lower activity than wild type (Fig 4A). O-glucosyltransferase activity was dependent on the concentrations of EGF repeat and UDP-glucose (acceptor and donor substrate, respectively). POGLUT1<sup>D233E</sup> also showed lower activity than wild type toward five different single EGF repeats from mouse Notch1 (Fig 4B), suggesting that the mutation affects O-glucosylation of all EGF repeats containing the O-glucose consensus sequence (CX5X(P/A)C) (Rana et al, 2011). Prior work has shown that POGLUT1 also has protein O-xyllosyltransferase activity toward certain EGF repeats with a diserine motif within the O-glucose consensus sequence (e.g. EGF16 of mouse Notch2) (Takeuchi et al, 2011). POGLUT1<sup>D233E</sup> mutant also showed lower O-xyllosyltransferase activity than wild type (Appendix Fig S7B). To examine whether POGLUT1<sup>D233E</sup> retains any enzymatic activity, we performed overnight incubation and then analyzed the products by reverse-phase HPLC and mass spectrometry. Both wild type and the D233E mutant added a single glucose or xylose to the hFIX-EGF repeat (Fig 4C) or EGF16 from mouse Notch2 (Appendix Fig S7C), indicating that POGLUT1<sup>D233E</sup> has residual enzymatic activity.

**Analysis of Notch signaling pathway and satellite cells in D233E patients**

Mutations in the Drosophila gene rumi, which encodes the fly-POGLUT1, result in a temperature-sensitive loss of Notch signaling (Acar et al, 2008). Moreover, Poglut1 knockdown in mouse myoblast C2C12 cells results in a significant decrease in O-glucosyltransferase activity and impaired Notch signaling (Fernandez-Valdivia et al, 2011). Accordingly, we examined the expression of activated Notch and the levels of Notch downstream targets in patient muscles. Decreases in Notch1-intracellular domain expression and in HES1 mRNA levels in skeletal muscle were detected compared with controls, which support that D233E affects Notch activation (Fig 5A and B). Notch signaling is critical in normal postnatal myogenesis by regulating SC maintenance (Bjornson et al, 2012), at least in part by directly inducing the expression of Pax7, which encodes a transcription factor critical for the maintenance of adult SC (Olguin & Olwin, 2004). Indeed, treating proliferating C2C12 cells with the γ-secretase inhibitor DAPT, which blocks Notch signaling, significantly reduced the mRNA levels of Pax7 (Appendix Fig S8). Likewise, we found a decrease in Pax7 mRNA and fewer Pax7<sup>+</sup> cells in the muscle of patients compared with controls (Fig 5C and D). Together, these observations suggest that the D233E mutation affects the self-renewal of SC by reducing Notch signaling, likely due to defective O-glucosylation.

We analyzed the same parameters in patients suffering from other muscular dystrophies, and also in patients suffering from a secondary dystroglycanopathy, whose muscle biopsies showed mild-to-moderate dystrophic changes, similar to our patients. Both groups show significantly higher levels of activated Notch and higher numbers of Pax7<sup>+</sup> cells compared with D233E patients (Appendix Fig S9A–H). These data support the notion that the observed decrease in Notch1 signaling and Pax7<sup>+</sup> cells in D233E patients is not secondary to α-dystroglycan hypoglycosylation.

**In vivo functional study of POGLUT1**<sub>D233E</sub>

To investigate the in vivo effects of the D233E mutation on POGLUT1 function in muscle progenitors, we performed cross-species rescue experiments in the context of adult myogenesis in Drosophila, a process regulated by Notch signaling (Gildor et al, 2012). In these experiments, we used the rumi<sup>79</sup> allele, which harbors a missense mutation that abolishes fly-POGLUT1 enzymatic activity but does not affect its expression (Acar et al, 2008). When raised at 18°C, rumi<sup>79/79</sup> animals exhibited normal indirect flight muscle development and a large number of myoblasts expressing the transcription factor Twist, which is a downstream target of Notch activation (Fig 6A–B). However, when rumi<sup>29/29</sup> animals were raised at 25 and 30°C during the early pupal stage, when these muscles form, muscle development was dramatically impaired and
the number of Twist$^+$ myoblasts was severely decreased (Fig 6C–D$^*$).

The severe temperature-sensitive defects observed in indirect flight muscle development in \textit{rumi$^{79/79}$} animals indicate that adult myoblast development in flies depends on the enzymatic activity of fly-POGLUT1. Overexpression of wild-type human-POGLUT1-FLAG in muscle progenitors rescued the muscle phenotypes of \textit{rumi$^{79/79}$}.

**Figure 3.** POGLUT1 D233E mutation in patients.

- **A** Genomewide mapping through high-density genotyping. The structural genomic variation and extended regions of homozygosity (loss of heterozygosity [LOH]) over the entire genome is shown for individuals II.1 to II.5. The homozygosity segment shared by the four affected siblings in chromosome 3 is indicated with an arrow.
- **B** This region is represented in more detail, where extended regions of homozygous genotypes in contiguous biallelic polymorphisms are highlighted with a green background.
- **C** The 14.6 megabase region of homozygosity that is shared by all affected siblings is highlighted by vertical red lines in (B), and genes within this genomic region are shown. Note that POGLUT1 gene is underlined.
- **D** Chromatograms reveal a homozygous T-to-G substitution (black boxes and arrowheads) in the four affected family members, while a heterozygous G/T in the healthy sibling.
- **E** Schematic structure of the human POGLUT1 protein, which contains a signal peptide (SP), a CAP10 domain, and a Lys-Asp-Glu-Leu (KDEL)-like endoplasmic reticulum retention signal. D233E is located in the CAP10 domain (arrow), close to the predicted ERD catalytic motif (asterisk). Alignment of amino acids flanking the mutated aspartic acid from POGLUT1 orthologs indicates evolutionary conservation of D233 in vertebrates (open arrowhead). Black boxes denote similarity of amino acid residues.
animals (Fig 6E). However, the overexpression of human-POGLUT1 \( \text{D233E-FLAG} \) only showed a weak and variable rescue of the muscle morphology and myoblast numbers in \( \text{rmut}^{79/79} \) animals (Fig 6F–H), indicating that this mutation significantly decreases the ability of POGLUT1 to promote muscle development and Notch signaling in myoblasts. It is interesting to note that fly-POGLUT1 harbors a glutamic acid (E) at position 252, which is equivalent to D233 in human-POGLUT1 (Fig 3E). Given the robust protein O-glucosyltransferase activity of the fly-POGLUT1 and because 35% of the amino acids in the CAP10 enzymatic domains of human and fly POGLUT1 are divergent (Acar et al., 2008), other amino acid differences between these two proteins are likely to be the reason why the fly protein is fully functional despite harboring the equivalent of the D233E mutation.

**Ex vivo analysis of D233E primary myoblasts**

Previous studies have shown that the Notch signaling pathway not only plays an important role in maintaining satellite cell quiescence, but also enhances myoblast proliferation and inhibits myogenic differentiation (Conboy & Rando, 2002; Bjornson et al., 2012; Mourikis & Tajbakhsh, 2014).

In order to directly test our hypothesis that the decrease in the POGLUT1 enzymatic activity caused by the D233E mutation disrupts muscle cell function, we explored myogenesis in D233E primary myoblasts from patient muscle biopsies compared with healthy and disease controls (Appendix Fig S10). We quantified the percentage of self-renewing, proliferating, and differentiating cells based on PAX7 and MyoD expression. Quantitative analysis in myoblasts cultured in a growth medium revealed that the percentage of PAX7\(^+\) MyoD\(^+\) (proliferating) cells in D233E cells was significantly lower than those in healthy and disease controls (Fig 7A–C). The percentage of PAX7\(^+\) cells and PAX7\(^+\) MyoD\(^-\) (self-renewing) cells was significantly decreased in D233E samples (Fig 7D and E), whereas the percentage of PAX7\(^+\) MyoD\(^-\) (differentiating) cells was increased (Fig 7F). When myoblasts reached confluence, they were cultured in a differentiation medium. We observed more efficient myoblast fusion into the multinucleated myotubes and higher levels of myogenin expression in D233E cultures compared with controls (Fig 7G–I). The slow proliferation and facilitated differentiation of mutant myogenic cells are in agreement with decreased Notch activity in the muscle of D233E patients.

Due to the scarcity of cells obtained from fresh patient’s muscles in culture (likely because of the severe defect in proliferation of D233E myoblasts), we performed immortalization of the primary myoblasts (Mamchaoui et al., 2011), and conversion of skin fibroblasts from patients to myogenic cells by transduction of inducible MyoD (Choi et al., 1990). Thus, we generated two different continuous myogenic cell lines with the D233E mutation, which showed similar phenotypic features, although PAX7 expression started after 1–3 days in culture in the myogenic cell line (Appendix Figs S11 and S12). To examine whether increasing Notch pathway activity can rescue the alterations of the myogenic process observed in D233E immortalized myoblasts during differentiation in culture, we used lentivirus to overexpress NICD1 in these cells and observed a solid reversion of the proliferation defects (Fig 8A and B, Appendix Fig S13A and B). However, we did not observe rescue of the amount of PAX7\(^+\) cells (Fig 8C). It has been described that the effect of Notch on the expression of PAX7 in myogenic cell lines such as C2C12 is not as potent as in primary myoblasts (Sun et al., 2008). This probably hindered the modification of PAX7 expression during the rescue experiment. In addition, NICD1 overexpression robustly rescued the facilitated differentiation shown by D233E myoblasts in culture (Fig 8D, Appendix Figs S13C and S14), and this...
rescue was demonstrated by the fusion index (Fig 8E) and expression of myogenin (Fig 8F). These results support the pathogenic role of decreased Notch signaling in this novel muscular dystrophy.

Role of Notch on $\alpha$-dystroglycan glycosylation

As shown in Fig 2, our patients exhibit a muscle-specific reduction in $\alpha$-dystroglycan glycosylation. The only known protein domain to which POGLUT1 can add carbohydrates is a properly folded EGF repeat containing the CX$\times$(P/A)C consensus sequence (Takeuchi et al, 2012). Since $\alpha$-dystroglycan contains no EGF repeats nor CX$\times$(P/A)C sites, it is unlikely to be a direct target of glycosylation by POGLUT1. A recent study has shown that in transgenic mice and C2C12 myoblasts, $\alpha$-dystroglycan glycosylation is a progressive process that initiates rapidly after myoblasts are induced to differentiate, and exhibits a regular and gradual transition to a larger glycoforms to reach the normal level after 5 days of differentiation (Goddeeris et al, 2013). In order to evaluate this progressive pattern of $\alpha$-dystroglycan glycosylation, primary immortalized myoblasts were differentiated. After 5 days with differentiation medium, D233E myoblasts showed a lower level and irregular progression profile of $\alpha$-dystroglycan glysosylation compared with controls, which reproduced the same pattern previously demonstrated in C2C12 cells (Goddeeris et al, 2013) (Fig 9A). These observations are compatible with abnormal differentiation dynamics in patient myoblasts. Given the well-established role of Notch signaling in

Figure 5. Muscles from D233E patients show decreased Notch signaling and pool of satellite cells.

A Western blot of muscle homogenates shows reduced expression of Notch1 intracellular domain (NICD) in patients. B, C qRT–PCR on RNA extracted from skeletal muscle shows that expression of HES1 (B) and PAX7 (C) was significantly lower in D233E patients compared with healthy controls. Mean ± SEM; Student’s t-test (B) and Mann–Whitney U-test (C). D PAX7$^+$ cells in skeletal muscle sections, demonstrating that satellite cells are less abundant in D233E patients than in controls ($n$ = 3 muscles with 10–15 fields analyzed per muscle). Mean ± SEM; Mann–Whitney U-test; scale bar, 50 µm.

Source data are available online for this figure.
muscle differentiation, we hypothesized that decreased Notch signaling in D233E patients’ myoblasts might contribute to altered α-dystroglycan glycosylation. We first attempted to test this hypothesis by asking whether NICD overexpression can restore α-dystroglycan glycosylation in patient myoblasts. However, given the strong inhibition of differentiation upon NICD overexpression,
Figure 7.

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containing DAPT or LY3039478 to decrease Notch signaling. The differentiation. To this end, we differentiated C2C12 cells with medium differentiating SC cells and muscular dystrophic Transgenic mice in which the pan-Notch inhibitor dnMAML1 is inactivation, produced by the reduced activity of POGLUT1 in the case of our patients.

**Discussion**

Transgenic mice in which the pan-Notch inhibitor dnMAML1 is specifically expressed in muscle SC were recently shown to display reduced PAX7 expression in SC cells and muscular dystrophic features even without experimentally induced injury (Lin et al., 2013). This study highlighted the role of Notch signaling and SCs in repairing the muscle damage caused by regular activity in a mammal, suggesting that a primary defect in SC maintenance caused by decreased Notch signaling can in principle result in muscular dystrophy. However, direct evidence from human patients was lacking to support this notion. Here, we describe a family in which a recessive D233E mutation severely impairs the enzymatic activity of POGLUT1, leading to a marked reduction in Notch activation in adult muscle, a decreased pool of PAX7+ SCs, and a partial defect of α-dystroglycan functional glycosylation. The findings in this family sharply contrast with those in other forms of age-matched limb-girdle muscular dystrophy, including secondary dystroglycanopathies, which show normal levels of HES1 and PAX7 transcripts in adult muscles (Appendix Fig S9).

Although patients’ muscles display reduced glycosylation of α-dystroglycan, several findings are inconsistent with a secondary dystroglycanopathy. First, the patients do not have elevated creatine kinase and muscle biopsies do not show many degenerative fibers compared with that observed in secondary dystroglycanopathies (Cirak et al., 2013). Second, the D233E muscles exhibit reduced α-dystroglycan laminin-binding activity but show normal agrin-binding and compact basement membrane ultrastructure. Although it is known that LARGE-dependent glycosylation of α-dystroglycan is required for the binding of both laminin and agrin, the precise glycan structures recognized by these ligands have not been determined. Agrin, but not laminin, showed normal binding to α-dystroglycan in patients’ muscle, suggesting that these ligands may recognize slightly different glycans structures on α-dystroglycan. Third, α-dystroglycan expression and laminin binding are normal in D233E skin fibroblasts. In contrast, other forms of dystroglycanopathies usually exhibit markedly diminished or no binding for both laminin and agrin in muscle, together with defective muscular basement membrane compaction (Michele et al., 2002; Haro et al., 2011; Willer et al., 2012; Goddeeris et al., 2013). Moreover, the published results show that secondary dystroglycanopathies caused by mutations in known genes result in reduced functional α-dystroglycan glycosylation in patient fibroblasts, and in the majority of patients, the level of α-dystroglycan glycosylation is comparable in fibroblasts and skeletal muscle from the same patient (Cars et al., 2013). These observations further support the notion that, although decreased laminin binding in muscle is likely to contribute to pathology in our...
Figure 8.
patients, α-dystroglycan hypoglycosylation might not be the disease mechanism caused by the D233E mutation, or at least the primary mechanism.

Strikingly, our data suggest that the D233E mutation causes defects in SC proliferation and differentiation. The amount number of SCs in our patients is significantly reduced even in very early stages when the muscle is barely affected, compared with healthy and disease controls. Alterations in SC proliferation have been described in the Largemyd mouse dystroglycanopathy model and are thought to contribute to myodegeneration in these animals (Ross et al., 2012). In this model, SCs are significantly increased at early stages and have reduced proliferation when cultured on freshly isolated single fibers; however, proliferation is restored in culture when SCs are removed from the fibers, indicating that rather than intrinsic defects in myoblasts, compositional changes in the extracellular matrix due to hypoglycosylated α-dystroglycan are responsible for the proliferative alteration. In contrast, myoblasts from our patients displayed reduced proliferation and self-renewal capacity in culture when removed from their fiber niche, supporting an intrinsic defect in D233E myoblasts independent of α-dystroglycan hypoglycosylation. Moreover, it has been recently demonstrated in dystroglycanopathy mice models that functional glycosylation of α-dystroglycan glycosylation during differentiation.

A Immortalized primary myoblasts from a healthy control showed a progressive pattern of α-dystroglycan glycosylation during 5 days of differentiation (D0–D5), as has been previously described by Goddeeris et al. (2013). Immortalized primary myoblasts from patients displayed an irregular pattern of glycosylation, with a final level of glycosylated α-dystroglycan that was lower than control.

B α-dystroglycan glycosylation of infected immortalized myoblasts from patient-LV-NICD-GFP at differentiation day 5 (D5) showed a striking reduction, in line with the strong inhibition of myotubes formation and differentiation induced by NICD overexpression. α-dystroglycan in myoblasts from patient-LV-GFP showed a reduction in glycosylation compared with control-LV-GFP at D5, similar to the results obtained in the adult muscle from patients (Fig 2).

C, D C2C12 myogenic cells showed a reduced level of glycosylated α-dystroglycan when the Notch signaling inhibitor DAPT or LY3039478 was added to the differentiation medium. This indicates that reducing Notch signaling can affect α-dystroglycan glycosylation in wild-type C2C12 myoblasts cells, which do not harbor any known mutations in α-dystroglycan glycosyltransferases or Poglut.

E, F qRT–PCR experiments show that upon DAPT or LY3039478 treatment, a remarkable decrease in Hes1 mRNA is induced in C2C12 cells during differentiation. Three replicates per condition were analyzed; mean ± SEM is shown.

Source data are available online for this figure.
α-dystroglycan does not play a significant role in myogenic cell proliferation and differentiation (Awano et al., 2015). Our results indicate that the D233E mutation results in deficient Notch signaling in patient muscle and an impairment in the regulation of myoblast differentiation and that expression of NICD rescues this phenotype. Our results strongly suggest that impaired myogenesis mediated by decreased Notch signaling is a causal mechanism of muscular dystrophy in our patients (Fig 8). Although we expected that the reduction in PAX7+ cells in our patients would be a direct effect of Notch inhibition, the lack of rescue of PAX7+ cells by NICD overexpression suggests something more complicated. The effect of Notch on PAX7 expression has been reported to be weaker in myogenic cell lines than in primary myoblasts, and this could be affecting the rescue of PAX7+ cells in our system (Sun et al., 2008). Nevertheless, our data consistently demonstrated a decreased number of PAX7+ cells in D233E patients, compared with healthy and disease controls, including secondary dystroglycanopathies. This result together with a previous report showing that the blockade of Notch signaling in muscle SCs of a mouse model causes muscular dystrophy and impairs muscle regeneration (Lin et al., 2013) supports our hypothesis that impaired regulation of muscle SC homeostasis plays a pathogenic role in this new muscle dystrophy. Recent findings demonstrate that the overexpression of Jagged1, a major ligand of the Notch signaling pathway, ameliorates the dystrophic phenotype in the golden retriever muscular dystrophic dogs, which is an excellent model to the human dystrophynopathy, indicating that the Notch pathway can be considered a new therapeutic target in muscular dystrophies (Vieira et al., 2015).

POGLUT1 specifically adds O-glucose or O-xylene to properly folded EGF repeats containing the CX3(S/P)/A/C consensus sequence (Rana et al., 2011; Takeuchi et al., 2011, 2012) and is among the glycosyltransferases that directly add sugar molecules to an amino acid, not to other sugars. Therefore, since α-dystroglycan lacks EGF repeats and the CX3(S/P)/A/C consensus sequence, POGLUT1 is highly unlikely to directly add carbohydrates to α-dystroglycan core protein or to extend the carbohydrate chains added to α-dystroglycan by other glycosyltransferases. This is supported by our observation that glycosylation of α-dystroglycan from D233E skin fibroblasts is normal. These data strongly argue that POGLUT1 does not participate in the biosynthesis of the O-mannose glycans on α-dystroglycan. Therefore, unlike POMT1/2 and LARGE which add carbohydrates to α-dystroglycan to regulate muscle maintenance, POGLUT1 most likely glycosylates other target proteins to maintain muscle integrity. This notion is supported by our fly experiments: Loss of POGLUT1 enzymatic activity in Drosophila also causes a severe decrease in the number of Twist+ myoblasts and muscle development, which can be fully rescued by wild-type human-POGLUT1 but only weakly so by POGLUT1D233E. However, Drosophila dystroglycanopathies models, including null alleles of dystroglycan, do not have defects in myoblast migration and myofiber formation (Nakamura et al., 2010), indicating that the muscle phenotypes observed in rumi flies are not caused by alterations in dystroglycan.

Fourteen Drosophila proteins and about 50 mammalian proteins have EGF repeats with a CX3(S/P)/A/C consensus sequence and are therefore predicted to be modified by POGLUT1 (Jafar-Nejad et al., 2010; Rana et al., 2011; Haltom et al., 2014). This consensus motif is highly predictive of enzymatic modification, as mass spectrometric analyses have demonstrated that most if not all EGF repeats with a CX3(S/P)/A/C motif examined so far are O-glucosylated (Hase et al., 1988; Acar et al., 2008; Fernandez-Valdivia et al., 2011; Rana et al., 2011; Lee et al., 2013; Haltom et al., 2014; Ramkumar et al., 2015; Thakurdas et al., 2016). Agrin, which is implicated in muscle disease, is predicted to have a single POGLUT1 target site. However, we did not observe any defects in agrin’s level, molecular size, or its capacity to bind α-dystroglycan in our patient muscles, strongly suggesting that impaired agrin glycosylation cannot explain the muscular dystrophy in our patients. Indeed, the addition of O-glucose to a target protein by POGLUT1 does not necessarily indicate functional importance. For example, even though Drosophila Crumbs is O-glucosylated, homozygosity for a knock-in allele of the Drosophila crumbs in which all seven Crumbs CX2-O-glucosylated, homozygosity for a knock-in allele of the Drosophila crumbs is predicted to be modified by POGLUT1, although further experiments are required to prove this (Thakurdas et al., 2016). Based on the severe decrease in Notch signaling and Notch1 cleavage in our patients’ muscle, the dramatic decrease in the ability of POGLUT1D233E to glycosylate Notch EGF repeats, and previous observations on the role of POGLUT1 and Notch receptor O-glucosylation in Notch signaling (Acar et al., 2008; Fernandez-Valdivia et al., 2011; Leonardi et al., 2011; Haltom et al., 2014; Ramkumar et al., 2015). Moreover, there is evidence suggesting that mouse JAG1 might also be a biologically relevant target of POGLUT1, although further experiments are required to prove this (Thakurdas et al., 2016). Based on the severe decrease in Notch signaling and Notch1 cleavage in our patients’ muscle, the dramatic decrease in the ability of POGLUT1D233E to glycosylate Notch EGF repeats, and previous observations on the role of POGLUT1 and Notch receptor O-glucosylation in Notch signaling (Acar et al., 2008; Fernandez-Valdivia et al., 2011; Leonardi et al., 2011; Ma et al., 2011; Rana et al., 2011), we propose that the Notch receptors are the key target of POGLUT1 in the muscle. However, we cannot exclude that other POGLUT1 targets might also contribute to the phenotypes observed in our patients.

If POGLUT1 affects a glycosylation pathway unrelated to the O-mannose glycans, it raises the question of why O-mannose glycosylation on α-dystroglycan is impaired in D233E muscle. Our data strongly favor an indirect mechanism for α-dystroglycan hypoglycosylation, likely due to altered myogenesis caused by decreased Notch signaling resulting from the D233E mutation. Indeed, a recent report showed that α-dystroglycan glycosylation is at a low level during the first stages of muscle regeneration even in wild-type mice and is increased as myogenesis proceeds (Goddeeris et al., 2013). The authors proposed that coordination between myogenic differentiation and increased expression of the glycosyltransferase LARGE results in bulkier α-dystroglycan molecules with more extensive LARGE-mediated glycosylation and thereby a more robust ligand-binding capacity during muscle repair (Goddeeris et al., 2013). Accordingly, α-dystroglycan hypoglycosylation in our patients can be a consequence of altered SC homeostasis and differentiation, which would impede muscle repair and potentially result in immature LARGE-mediated glycosylation (Fig 9). Previous data show that an adequate balance of Notch activation is needed during myogenesis (Kuang et al., 2007; Sacco et al., 2008), and our results support an additional critical role of Notch signaling in the process of α-dystroglycan glycosylation during muscle differentiation. Nonetheless, the muscle-specific α-dystroglycan hypoglycosylation could contribute to muscle dystrophy in our patients.

An autosomal-dominant dermatosis has recently been associated with heterozygous mutations in POFUT1 (protein O-fucosyltransferase
1, which adds O-fucose to Notch), and POGLUT1 (Li et al, 2013; Basmanav et al, 2014). Drosophila Pofut1 has a chaperone-like function on the Notch protein independent of its O-fucosyltransferase activity (Okajima et al, 2005). Therefore, since patients homozygous for D233E have deficient enzymatic activity but normal POGLUT1 expression and no skin abnormalities, this dermatosis could be due to a defect in a non-enzymatic function of POGLUT1. However, genetic background differences could also exist that make these patients more sensitive to a 50% reduction in POGLUT1 enzymatic activity.

In conclusion, we identified the human POGLUT1 D233E mutation that impairs Notch posttranslational modification and maintenance of muscle stem cells and leads to muscular degeneration and α-dystroglycan hypoglycosylation. Our findings demonstrate that D233E mutation in POGLUT1 causes autosomal recessive limb-girdle muscular dystrophy and implicate a primary defect in muscle progenitor cells as a novel pathomechanism for muscular dystrophy. Our patients display an adult-onset pure muscular phenotype, in contrast to the lethal, multisystemic muscular dystrophy. Our patients display an adult-onset pure muscular phenotype, in contrast to the lethal, multisystemic muscular dystrophy. Our patients display an adult-onset pure muscular dystrophy. Our patients display an adult-onset pure muscular dystrophy. Our patients display an adult-onset pure muscular dystrophy.

Materials and Methods

Skeletal muscle histology and ultrastructural study

Open muscle biopsies were obtained from biceps brachii or quadriceps of the four patients and the healthy sibling in generation II of the family. The muscle was rapidly frozen in liquid nitrogen-chilled isopentane and 7-μm-thick cryostat sections were stained for standard histological and histochemical techniques including hematoxylin and eosin, Comori trichrome, nicotinamide adenine dinucleotide dehydrogenase (NADH), and ATPase pH 9.4. Stained sections were evaluated with an Olympus BX41 (Tokyo, Japan) equipped with a ColorView IIIu camera (Olympus).

A piece of muscle from each biopsy was fixed in glutaraldehyde, embedded in Embed 812 resin, and processed for electron microscopy by standard methods (Malfatti et al, 2013). Ultrathin sections were viewed with Philips CM10 electron microscope (Eindhoven, Netherlands) equipped with Gatan 1k CCD camera.

Antibodies

The list of the primary antibodies used in this study is provided in Appendix Table S7. The anti-α-dystroglycan core protein (a kind donation from S. Kröger) sheep polyclonal antibody was raised against a synthetic peptide corresponding to the carboxy-terminal 20 amino acids of chick α-dystroglycan (Herrmann et al, 2000).

Immunohistochemical studies

Unfixed frozen muscle sections were incubated with primary antibodies overnight at 4°C, and then with the appropriate secondary antibodies for 30 min.

Cultured myoblasts were permeabilized in 0.2% Triton X-100 for 10 min and incubated in 1% BSA/PBS for 45 min; frozen sections were incubated in 5% normal goat serum/PBS for 45 min. Primary antibodies were used as described in Appendix Table S7 and incubated for 12 h at 4°C. After washing, samples were incubated with the appropriate secondary antibodies for 1 h.

PAX7 immunohistochemistry was carried out according to an antigen retrieval protocol, modified from Song et al (2012). Briefly, samples were fixed with 4% paraformaldehyde, pH = 7.4 (PFA) for 15 min. Sections were then immersed in citrate buffer (pH 6) for 30 min at 80°C and washed with PBS. Afterward, tissue samples were blocked with non-fat milk at 2% in PBS for 30 min and washed with PBS. Next, tissue sample were incubated with primary antibody for 2 days and secondary antibody for 2 h in blocking solution (5% BSA, 0.5% Triton X-100 in PBS). αDG-CORE immunohistochemistry required incubation with both biotinylated secondary antibody for 30 min and streptavidin conjugated to Cy3 for 15 min in PBS. Finally, the nuclei were stained for 20 min with To-pro-3-Iodide (Topro) at 1:1,000 in PBS and the slides were covered-slipped with fluorescence mounting medium (Dako). The images were acquired on a Zeiss LSM 710 confocal laser scanning microscope. Maximal projections of Z-stacked images were obtained and analyzed with ImageJ software.

Glycoprotein enrichment and Western blot analysis

Frozen muscle samples and fresh skin fibroblasts were homogenized in RIPA buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL, 0.1% SDS) containing protease inhibitor mixture. Western blots of glycoproteins were enriched with wheat-germ agglutinin (WGA) agarose (Sigma-Aldrich) as described previously (Michele et al, 2002). Equivalent amounts of protein lysates non-incubated or incubated with WGA agarose beads were resolved on 7–10% SDS-PAGE gels and transferred to PVDF membranes (Millipore). Immunoreactivity was detected with secondary antibodies conjugated to horseradish peroxidase (Jackson Immuno Research) and developed with SuperSignal West Femto (Thermo Scientific) using an ImageQuant LAS 4000 MiniGold System (GE Healthcare).

Ligand overlay assay

Ligand overlay assays in muscle and skin fibroblasts were performed as previously described with minor modifications (Michele et al, 2002; Willer et al, 2012). Briefly, PVDF membranes were incubated with Engelbreth-Holm-Swarm laminin (Sigma-Aldrich) or agrin (R&D Systems), overnight at 4°C in laminin-binding buffer. Then, membranes were washed and incubated with anti-agrin (Millipore) or anti-laminin (Sigma-Aldrich) primary antibodies and their corresponding secondary antibodies. Blots were imaged using the protocol described for Western blots.

Flow cytometry

The following method was modified from Stevens et al (2013b). Briefly, an anti-α-dystroglycan antibody IIH6 was used to assess the amount of α-dystroglycan glycosylation in fibroblasts from patients (II.4 and II.5) and two controls. Fibroblasts (passages 2–3) were
grown until approximately 90% confluent. Cells were detached using Accutase (Sigma, UK), centrifuged for 3 min at 500 g, and counted and resuspended in PBS to a final density of 200,000 fibroblasts per milliliter, centrifuged at 3,000 g for 3 min, and incubated on ice with IHH6 for 30 min, anti-mouse biotinylated IgM (Vector Labs, USA) for 20 min, and streptavidin-PE (BD Pharmingen, UK) for 15 min. After washing, cells were resuspended in 500 ml of PBS and transferred to FACS tubes (Corning Science, Med). Data were acquired using the BD FACS Canto II analyzer and analyzed using the BD FACSDiva software (BD Bioscience, US). Two separate controls for each fibroblast population were used: one without any staining to remove background and another without the primary antibody to gate the IHH6-positive population. A total of 50,000 cells were analyzed per experiment. We assessed the percentage of IHH6-positive cells as well as the level of IHH6 the mean fluorescence intensity (MFI).

Genetic mapping

Genomewide genotyping using the Illumina Infinium HumanOmni1-Quad, v1.0 BeadChip (Illumina, San Diego, CA, USA) was performed in the DNA samples of the four affected siblings (II.1, II.2, II.4, and II.5) and the unaffected sister (II.3), thus allowing the analysis of over one million assays for each sample. The chips were scanned, and data were loaded into Illumina Genome Studio V2009.1 software. Genome Viewer tool was used to visualize genomic copy number variation as well as individual genotypes. Log R ratio and B allele frequency, plotted along the entire genome for all SNPs on the array, were used to analyze all variants. CNV partition 2.4.4 was employed to automatically detect copy number variants as well as regions of extended homozygosity > 500 kb across the genome. Genotype success rates > 99.7% were obtained for all assays (Appendix Fig S6).

Exome sequencing

The capture and subsequent sequencing of exonic regions of human samples II.3, II.4, and II.5 was performed at Otogenetics Co (Norcross, GA, USA) using the solution-based SeqCap EZ Human Exome Library v2.0 (Roche NimbleGen, Madison, WI, USA) and the Illumina HiSeq2000, with 100 paired-end runs. An average coverage of 30X across the exome was achieved for all samples. Reads were aligned to the reference genome hg19 with DNAxenix software (Palo Alto, CA, USA) using default parameters. Single nucleotide polymorphisms and indels were identified and realigned using the Genome Analysis Toolkit (GATK). In order to discard common variants, genetic variations were further verified in the Database of Single Nucleotide Polymorphisms (dbSNP Build ID: 137; http://www.ncbi.nlm.nih.gov/SNP/), the exome variant server (EVS) of the National Heart, Lung, and Blood Institute GO Exome Sequencing Project (Seattle, WA, USA; http://evs.gs.washington.edu/EVS/), and the 1000 Genomes Project (http://www.1000genomes.org/).

Segregation analysis

Segregation analysis was performed by means of Sanger sequencing using the primers presented in Appendix Table S4. Cycle sequencing was performed using the Dye Terminator Sequencing Kit (Applied Biosystems, Foster City, CA) and run on an ABI 3100 genetic analyzer. Sequence chromatograms were analyzed using the Sequencher software (GeneCodes, Ann Arbor, MI).

Genotyping

Genotyping of the p.D233E mutation in POGLUT1 gene in the Spanish and the Human Genome Diversity Project series was carried out by real-time PCR using a custom TaqMan® Genomic Assay (Applied Biosystems, Foster City, CA) with appropriate primers and probes (Appendix Table S5). Thermal cycling and end-point polymerase chain reaction (PCR) analysis was performed on a real-time PCR instrument (ABI 7900HT, Applied Biosystems, Foster City, CA). DNA from family members II.3 and II.4 was used as positive controls in all experiments.

POGLUT1 subcloning and mutagenesis

Human POGLUT1 cDNA cloned into the pEZ-M02 vector was obtained from GeneCopoeia (Rockville, MD, USA). The p.D233E mutation was introduced with the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA), as per manufacturer’s indications, and confirmed by direct sequencing. The oligonucleotide sequences used for site-directed mutagenesis and Sanger sequencing were as follows: POGLUT1 5’-CACCTCTCCG GACCTCAGC-3’ and 5’-TAATAAGCAGCTACTATAAG3’ for wild-type and POGLUT1 5’-TCTCGGAAAAACCCAAAACTTGTGAGGC AGAATACACAAA-3’ and 5’-TTTGTTGTATCTGTGCTAACAAGTT TTGGGTCCCCGAGA-3’ (mismatched nucleotide to the reference sequence is underlined) for the D233E mutant. cDNAs encoding the luminal domains of wild type and D233E mutant of human POGLUT1, starting from Arg24, were amplified by PCR using the primers, 5’-ATATATATGCTCAGAGCAGAGTACAGT-3’ and 5’-ATCTACCTCGAGTCTAGCTACATTTTG-3’, and subcloned into a pSecTag2c vector (Invitrogen) in frame with a C-terminal Myc/6xHis tag for expression in mammalian cells. The expression constructs for wild-type human POGLUT1 in pcDNA4 or pTracer vector were also used as a template for site-directed mutagenesis to introduce the D233E mutation (Rana et al, 2011; Takeuchi et al, 2012). The sequences of the primers are 5’-CT GGTTGAGCAGAACACACAAAAACCAGGCTG-3’ and 5’-GTATT CTGCCCTCAACTAGTCTGAGGTTCTCGGAGACAG-3’. The pcDNA4-based construct encodes the recombinant protein with a C-terminal Myc/6xHis tag. The pTracer-based construct encodes the recombinant protein with a FLAG tag, which is immediately downstream of the KDEL sequence at the C-terminus. Successful incorporation of the mutation was confirmed by direct DNA sequencing.

In vitro glycosylation assays

Myc/6xHis-tagged POGLUT1 proteins were expressed in HEK293T cells by transient transfection, and the secreted proteins were purified from the culture media using Ni-NTA agarose (QIAGEN). POGLUT1/POXYLT assays were performed as previously described (Fernandez-Valdivia et al, 2011). Briefly, a 10 μl standard reaction mixture contained 50 mM HEPES pH 6.8, 10 mM MnCl2, the indicated amounts of acceptor substrates, 0.16 μM UDP-[6-3H]glucose
(2.22 TBq/mmol), 10 μM UDP-glucose, 0.5% Nonidet P-40, and purified proteins. For POXYLT assay, 10 μM UDP-[14C(U)]xylose (5.43 GBq/mmol) was used as donor substrate. The reaction was performed at 37°C for 20 min and stopped by adding 900 μl of 100 mM EDTA pH 8.0. The sample was loaded onto a C18 cartridge (100 mg, Agilent Technologies). After the cartridge was washed with 5 ml of H2O, the EGF repeat was eluted with 1 ml of 80% methanol. Incorporation of [6-3H]glucose or [14C(U)]xylose into the EGF repeats was determined by scintillation counting of the eluates. Reactions without substrates were used as background control. Data are from three independent assays. The values indicate mean ± SEM.

O-glycosylation or O-xylosylation of human factor IX EGF repeat and EGF16 from mouse Notch2 was performed using a modified POGLUT/POXYLT assay. A 100 μl reaction mixture contained 50 mM HEPES pH 6.8, 10 mM MnCl₂, 10 μM acceptor substrate, 200 μM UDP-glucose, and purified wild-type or D233E mutant POGLUT1. For O-xylosylation, 200 μM UDP-xylose was used as donor substrate. Approximately 500 ng of each recombinant enzyme was used. The reaction was performed at 37°C overnight. The reaction products were purified by reverse-phase high-performance liquid chromatography (RP-HPLC, Agilent Technologies, 1200 Series) equipped with a C18 column (10 × 250 mm, VYDAC) with a linear gradient of solvent B (80% acetonitrile, 0.1% trifluoroacetic acid (TFA) in water) from 10 to 90% in solvent A (0.1% TFA in water) for 60 min, monitoring absorbance at 214 nm. Elution profiles between 20 and 30 min are shown as milli-absorbance unit (mAU). Peak fractions were pooled, dried down in a Speed-Vac centrifuge, and stored at −20°C for further mass spectral analysis.

Mass spectrometric analysis of glycosylation products

Mass spectrometric analysis by infusion was performed as described previously (Takeuchi et al., 2012). Briefly, dried samples were resuspended in 0.1% formic acid in water, spin-filtered, and injected into an Agilent 6340 ion-trap mass spectrometer with a nano-HPLC CHIP–Cube interface at a rate of 18 μl/h. The MS peaks for MS/MS top of peaks in HPLC profiles. Mass spectrometric analysis of glycosylation products by infusion was performed as described previously (Takeuchi et al., 2012). Briefly, dried samples were resuspended in 0.1% formic acid in water, spin-filtered, and injected into an Agilent 6340 ion-trap mass spectrometer with a nano-HPLC CHIP–Cube interface at a rate of 18 μl/h. The MS peaks for MS/MS were chosen manually, and the data were analyzed using Agilent ChemStation data analysis software. The masses of EGF repeats with different charge states were deconvoluted and shown on the top of peaks in HPLC profiles.

RT–PCR and qRT–PCR analysis

Total RNA was extracted using QIAzol and RNeasy kit (Qiagen). cDNA was synthesized from 1 μg of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche). RT–PCR (100 ng total cDNA per reaction) was performed using TaqMan® Fast Advanced Master Mix (Applied Biosystems) in a 7500 Fast Real-Time PCR System. Relative mRNA levels were compared using the 2−ΔΔCt method, with GAPDH as control (only POGLUT1 mRNA level was relative to desmin). We used TaqMan probes (Applied Biosystems) for human HES1 (Hs01727878_m1), human PAX7 (Hs00242962_m1), human POGLUT1 (Hs00220308_m1), human GAPDH (Hs02758991_g1), human DES (Hs00157258_m1), mouse HES1 (Mm01342805_m1), mouse PAX7 (Mm01354484_m1), and mouse GAPDH (Mm03302249_g1).

We selected the disease control muscles from patients with well-characterized limb-girdle muscular dystrophies. To avoid possible differences due to the effect of age on the satellite cell population (Chakkalakal et al., 2012; Sousa-Victor et al., 2014), our samples of D233E patients and both healthy and disease controls were age-matched (Appendix Table S6).

Generation of the transgenic fly

To generate transgenic flies capable of overexpressing human POGLUT1WT, a BamHI-XbaI fragment containing the POGLUT1WT ORF was released from pTracer-POGLUT1WT, FLAG and cloned into the BglII-XbaI sites of pUASattB (Bischof et al., 2007) to generate pUASattB-POGLUT1WT-FLAG. Generation of pUASattB-POGLUT1WT-FLAG was described previously (Fernandez-Valdivia et al., 2011). Both constructs were integrated into the VK22 docking site by using FlpC-mediated transgenesis to generate UASattB-POGLUT1WT-FLAG.VK22 and UASattB-POGLUT1WT-FLAG.VK22 transgenes. Correct integration events were verified by att PCR, as described previously (Venken et al., 2006).

Analysis of muscle phenotype in Drosophila

We assessed the effect of POGLUT1 activity toward the development of indirect flight muscles in Drosophila (Gildor et al., 2012). Rescue experiments were performed with fly transgenes expressing human POGLUT1wt (wild type) and human POGLUT1D233E wt118 (wt), rumi79/79, MeF2-GAL4/UASattB-POGLUT1WT-FLAG; rumi79/79, and MeF2-GAL4/UASattB-POGLUT1D233E-FLAG; rumi79/79 animals were raised at 18°C until puparium formation. Pupae 0–1 h after puparium formation (APF) were incubated at indicated temperatures until 25% pupal development. Pupal indirect flight muscles were dissected and stained using standard methods. Antibodies are rabbit α-Twist 1:5,000 (Roth et al., 1989), mouse 22C10 1:50 (DSHB), mouse α-FLAG M2 1:100 (Sigma), donkey-α-rabbit-Cy3 1:500, donkey-α-mouse-Cy5 1:500 (Jackson ImmunoResearch Laboratories). Confoal images were scanned using a Leica TCS-SP5 microscope and processed with Amira5.2.2. Myotube lengths were measured using a two-dimensional measurement tool in Amira5.2.2. Mean myotube lengths and standard deviations were calculated for rumi79/79 pupae with no rescue and rumi79/79 pupae overexpressing POGLUT1WT (n = 22) or POGLUT1D233E (n = 54). One-way ANOVA with Tukey’s multiple comparisons test was used to determine the P-values. Images were processed with Adobe Photoshop CS5; figures were assembled in Adobe Illustrator CS5.

C2C12 cell line culture and Notch inhibition assay

C2C12 cell line was cultured in monolayer. The culture media for the C2C12 myoblast stage contains Dulbecco’s minimal essential medium (DMEM, Gibco, BRL, Bethesda, MD), supplemented with 5% glucose, 10% fetal bovine serum, 1% penicillin-streptomycin-fungizone, and 23 mM HEPES. The cultures were examined regularly to avoid overgrowth. For the Notch inhibition assay, we treated C2C12 myoblasts at 80–90% confluence with DAPT (Sigma-Aldrich, USA), a γ-secretase inhibitor, and a well-established inhibitor of Notch, a γ-secretase substrate, as previously published (Kuang et al., 2007). We also treated C2C12 myoblasts with LY3039478 (ApexBio Technology LLC), a more specific and potent Notch inhibitor. For this experiment, culture proliferation and
differentiation medium were modified by adding 50 μM DAPT or 25 μM LY3039478, and 50–25 μM DMSO for controls, respectively.

Primary cell culture

Muscle biopsies from two patients (II.4 and II.5), three age-matched healthy controls and three age-matched disease controls were used for the experiments. Muscle biopsies were minced and cultured in a monolayer. Briefly, the culture medium for the myoblast stage contains 75% Dulbecco’s minimal essential medium (Invitrogen) and 25% M199 medium (Invitrogen), supplemented with 10% fetal bovine serum (FBS), 10 μg/ml insulin, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 10 ng/ml epidermal growth factor, and 25 ng/ml fibroblast growth factor. To obtain highly purified myoblasts, each 107 cells were mixed with 20 μl of CD56-coated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated at 6°C–12°C for 15 min. Unbound microbeads were removed by washing cells in excess PBS buffer followed by centrifugation at 300 × g for 10 min. The cell pellet was resuspended in PBS buffer to a concentration of 2 × 106 cells/ml before separation on a midiMACS cell separator (Miltenyi Biotec, Bergisch Gladbach, Germany). CD56-positive cells were seeded at 13,000 cells/cm² using the culture medium for the myoblast stage containing 15% of FBS and without growth factors to avoid interference with the results. We examine proliferation at different days. When the myoblasts started to fuse, the medium was substituted with one containing 2% of FBS. We measured the fusion index 4 days after medium change by calculating the mean percentage of nuclei in myotubes in respect to the total number of nuclei (myoblasts + myotubes).

Human skin biopsies were minced and cultured. Fibroblasts were grown in DMEM containing 15% fetal bovine serum and 1% penicillin-streptomycin-fungizone at 37°C with 5% CO2.

Myoblast immortalization

Myoblasts from II.4 patient and healthy control were transduced with lentiviral vectors encoding hTERT and cdk4 containing puromycin and neomycin selection markers, respectively. Transduced cells were selected with puromycin (1 μg/ml) for 6 days and neomycin (1 mg/ml) for 10 days. Infected cells were purified using an immunomagnetic cell sorting system (MACS; Miltenyi) according to the manufacturer’s instructions with anti-CD56 microbeads. Cells were seeded at clonal density, and individual myogenic clones were isolated and characterized.

Fibroblast myogenic conversion

Fibroblasts from II.4 patient and healthy control were transduced with lentiviral vector encoding hTert. Transduced cells were selected with puromycin (1 μg/ml) for 6 days. Immortalized fibroblasts were then transduced with a lentiviral vector encoding inducible MyoD. For MyoD induction, doxycycline (2 μg/ml) was added in the proliferation medium (this day was considered day 0).

Lentivirus generation and infection

We generated lentiviral vectors (LV), based on the calcium phosphate method as previously described (Yoshida et al, 2013). Briefly, HEK293T cells were transfected using transfer plasmids, EF.hICN1.CMV.GFP (#17623, Addgene) or EF.CMV.GFP, together with pSPAX2 packaging and pMD2.G envelope plasmid DNA at a ratio of 4:3:1, respectively. Then, 48 h post-transfection, medium was collected, filtered, and concentrated by centrifugation for 90 min at 105,000 × g using a SW 32 Ti Beckman rotor. Supernatant was completely removed and pellet diluted in 100 μl PBS overnight at 4°C, titered by FACS, and stored at −80°C until use. Immortalized myoblasts from patients were infected with LV-NICD-GFP or LV-GFP, and immortalized myoblasts from control with LV-GFP. After 7 days, GFP-positive cells were purified by FACS.

Study approval

This study was approved by the Institutional Research Ethic Committee at Hospital Universitari Virgen del Rocío in Sevilla (Spain). Written informed consent was received from participants, prior to inclusion in the study, for genetic studies, for muscle and skin biopsies, and for pictures appearing in the manuscript.

Expanded View for this article is available online.

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Author contributions

CP and JC designed the study. ES-M performed α-dystroglycan expression and function studies, cell culture, satellite cells, and myogenesis analysis. HT performed biochemical in vitro assays. TLV and BE performed the fly studies. CP, MC-S, YM, and CM handled patients and collected biological samples from the patients. ER and ES-M processed and studied muscle biopsy. CP, MC-S, and JAM-L analyzed the radiological findings. FM, CP, JLN-G, and LG-S analyzed protein expression and mRNA levels. JC and GP performed the function studies, cell culture, satellite cells, and myogenesis analysis. HT, MC-S, and JAM-L analyzed the radiological findings. FM, CP, JLN-G, and LG-S analyzed protein expression and mRNA levels. JC and GP performed the function studies, cell culture, satellite cells, and myogenesis analysis.
research. CP, MH, RSH, and H-JN supervised and mentored all work. CP coordinated all the study and wrote the initial manuscript. All authors contributed to the final version of the manuscript.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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