High N-glycan multiplicity is critical for neuronal adhesion and sensitizes the developing cerebellum to N-glycosylation defect

Daniel Medina-Cano1,2, Ekin Ucuncu1,2, Lam Son Nguyen1,2, Michael Nicouleau1,2, Joanna Lipecka3, Jean-Charles Bizot4, Christian Thiel5, François Foulquier6, Nathalie Lefort7, Catherine Faivre-Sarrailh8, Laurence Colleaux1,2, Ida Chiara Guerrera3, Vincent Cantagrel1,2*

1Paris Descartes-Sorbonne University, Paris, France; 2Developmental Brain Disorders Laboratory, Imagine Institute, INSERM UMR 1163, Paris, France; 3Proteomics platform 3P5-Necker, Université Paris Descartes - Structure Fédérative de Recherche Necker, INSERM US24/CNRS UMS3633, Paris, France; 4Key-Obs SAS, Orleans, France; 5Center for Child and Adolescent Medicine, Kinderheilkunde I, University of Heidelberg, Heidelberg, Germany; 6Université Lille, UMR 8576 – UGSF - Unité de Glycobiologie Structurale et Fonctionnelle, CNRS, Lille, France; 7iPS Core Facility, Imagine Institute, Paris, France; 8Aix Marseille University, INSERM UMR1249, Marseille, France

Abstract Proper brain development relies highly on protein N-glycosylation to sustain neuronal migration, axon guidance and synaptic physiology. Impairing the N-glycosylation pathway at early steps produces broad neurological symptoms identified in congenital disorders of glycosylation. However, little is known about the molecular mechanisms underlying these defects. We generated a cerebellum specific knockout mouse for Srd5a3, a gene involved in the initiation of N-glycosylation. In addition to motor coordination defects and abnormal granule cell development, Srd5a3 deletion causes mild N-glycosylation impairment without significantly altering ER homeostasis. Using proteomic approaches, we identified that Srd5a3 loss affects a subset of glycoproteins with high N-glycans multiplicity per protein and decreased protein abundance or N-glycosylation level. As IgSF-CAM adhesion proteins are critical for neuron adhesion and highly N-glycosylated, we observed impaired IgSF-CAM-mediated neurite outgrowth and axon guidance in Srd5a3 mutant cerebellum. Our results link high N-glycan multiplicity to fine-tuned neural cell adhesion during mammalian brain development.

DOI: https://doi.org/10.7554/eLife.38309.001

Introduction

Protein N-glycosylation, one of the most abundant post-translational modification, helps direct various cellular functions, such as protein folding, stability, trafficking and localization (Cherepanova and Gilmore, 2016) (Freeze et al., 2014). Nearly all proteins transported through the secretory pathway undergo N-glycosylation, particularly to regulate cell surface abundance and cellular interactions (Dennis et al., 2009). This template-independent process shows distinct glycosylation patterns that vary by protein and physiological context (Dennis et al., 2009). Protein N-glycosylation begins in the endoplasmic reticulum (ER), where a tightly controlled and conserved biosynthetic pathway

*For correspondence: vincent.cantagrel@inserm.fr
Competing interests: The authors declare that no competing interests exist.
Funding: See page 21
Received: 11 May 2018
Accepted: 01 October 2018
Published: 12 October 2018
Reviewing editor: David D Ginty, Harvard Medical School, United States

Copyright Medina-Cano et al. This article is distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use and redistribution provided that the original author and source are credited.
synthesizes a precursor named the lipid-linked oligosaccharide (LLO). This LLO comprises a sequential assembly of 14 monosaccharides (Glc3Man9GlcNAc2) on top of the phosphorylated lipid carrier dolichol. Then, the oligosaccharyltransferase (OST) complex transfers en bloc the oligosaccharide part of the LLO to Asn residues on specific sites (Asn-X-Ser/Thr, X≠Pro) in nascent glycoproteins (Chavan and Lennarz, 2006). N-linked glycan undergoes final modifications in the ER and Golgi compartments.

Disrupting the N-glycosylation process in humans causes congenital disorders of glycosylation (CDG), a wide and highly heterogeneous group of inherited metabolic disorders (Ng and Freeze, 2018) (Freeze et al., 2014) (Jaeken and Péan, 2017). The analysis of the clinical and metabolic consequences of each defect is challenging as the underlying mutated enzymes or transporters are often involved in multiple and intricate pathways(Freeze et al., 2014). Among them, disrupted synthesis or transfer of LLO underlies the most prevalent disorder, CDG type I (CDG-I)(Jaeken and Péan, 2017). CDG-I diagnosis is based on the detection of hypoglycosylation of the patients’ serum transferrin that exhibits unoccupied N-glycosylation site(s). Clinically, CDG-I disorders present psychomotor retardation (the most common feature) associated with cerebellar ataxia, seizures, retinopathy, liver fibrosis, coagulopathies, abnormal fat distribution and failure to thrive. Cerebellar ataxia is the primary diagnostic indicator in patients with PMM2 mutations (PMM2-CDG, also known as CDG-Ia disorder), which is the most frequent CDG (Schiff et al., 2017). These phenotypic defects may arise from unoccupied glycosylation sites (i.e. protein hypoglycosylation) in numerous and mostly unidentified proteins.

The efficacy of protein glycosylation relies on the primary sequence at the glycosylation site (i.e. the sequon), the neighboring amino acids and the local structure (Poljak et al., 2018) (Murray et al., 2015). In CDG, the limited amount of LLO, the OST complex substrate, can also impact glycosylation site occupancy to cause hypoglycosylation (Birch and Aebl, 1999) (Freeze et al., 2015b). Initial studies investigated the consequences of this metabolic defect mainly in serum (Hülsmeier et al., 2007) (Richard et al., 2009). However, tissue accessibility and technical limitations precluded examining defects in organs, specifically the developing brain (Freeze et al., 2015b). The lack of data related to the consequence of the defect at the proteomic level contrasts with the well-known importance of N-glycans in specific phases of neural development (Scott and Panin, 2014b).

While several CDG models showing neural developmental defects exist, the mis-glycosylated targets remain unidentified (Chan et al., 2016; Cline et al., 2012; Scott and Panin, 2014a). We still do not know whether some specific proteins show a predisposition for these alterations, which contribute to disease pathogenesis. We predict that some glycoproteins possess a proclivity for hypoglycosylation based on their intrinsic properties (Poljak et al., 2018) (Hülsmeier et al., 2007). Previous studies identified SRD5A3, an enzyme involved in LLO synthesis, as mutated in CDG type I patients (SRD5A3-CDG) (Cantagrel et al., 2010) (Morava et al., 2010). SRD5A3 is a steroid-reductase-like enzyme involved in the last step of de novo synthesis of dolichol, the lipid used to build the LLO precursor. The broad clinical spectrum observed in patients with SRD5A3 mutations shows many similarities with other ER-related glycosylation defects including psychomotor retardation and cerebellar ataxia. These symptoms result from likely null SRD5A3 alleles (Kara et al., 2014; Tuysuz et al., 2016) (Wheeler et al., 2016) (Cantagrel et al., 2010) (Gründahl et al., 2012) and reflect impaired protein N-glycosylation, as previously described in yeast and mouse embryos mutated for the corresponding SRD5A3 ortholog (Cantagrel et al., 2010). Here, we sought to gain mechanistic and functional insight into protein N-glycosylation during neural development, so we used conditional disruption of the Srd5a3 gene in the mouse cerebellum, a tissue often affected in CDG.

Results

Conditional deletion of Srd5a3 in the whole developing cerebellum

Since we sought to examine the function of SRD5A3 in brain disease pathogenesis, we generated targeted conditional and null alleles of mouse Srd5a3, flanking exons 2 – 4 with loxP sequences (Figure 1—figure supplement 1A,B). Homozygous germline mutants (Srd5a3<sup>−/−</sup>) are embryonic lethal (data not shown) consistent with results from the Srd5a3 gene-trap mutant (Cantagrel et al., 2010). So, we used the Engrailed1-cre (En1-Cre) transgenic line to produce conditional knockouts En1-Cre;
Srd5a3<sup>fl-/</sup> in the developing hindbrain (Sgaier et al., 2007) and confirmed the gene deletion at the transcript level (Figure 1—figure supplement 1C–E). En1-Cre; Srd5a3<sup>fl-/</sup> mice were fertile and showed nearly Mendelian ratios at weaning age (data not shown). We used far-western blotting (far-WB) with biotinylated Sambucus Nigra lectin (SNA) to investigate the abundance of complex sialylated N-glycans (Cao et al., 2013). Total protein extracts from mutant cerebellums showed a non-significant 12% decrease in normalized signal intensity (Figure 1A,B). We obtained similar results with Concanavalin A (ConA) lectin that binds to core and immature mannosylated N-glycans (Figure 1—figure supplement 1F,G). These results indicate that cerebellar Srd5a3 deletion does not cause a severe general glycosylation defect. However, Lamp1, a broadly used marker for N-glycosylation defects (Rujano et al., 2017) (Kretzer et al., 2016), indicated a severe decrease in protein levels across different tissues (Figure 1C–E). The remaining Lamp1 showed a shift toward a lower molecular weight before PNGase treatment, which indicated impaired glycosylation (Figure 1D,F).

Next, we investigated the functional consequences of this cerebellar glycosylation defect on learning ability and motor behavior (Koziol et al., 2014) in a cohort of control and mutant mice (n = 30). In the Morris Water Maze (MWM), we exposed mice twice to the same hidden platform to test working memory. We found no significant difference on swimming speed between En1-Cre; Srd5a3<sup>fl-/</sup> and control mice. In a rotarod test, we observed a significant decrease in the latency to fall for the mutant compared to control (Figure 1G). These results suggest that the cerebellar glycosylation defect affects motor function.

**Figure 1.** Deletion of Srd5a3 in the cerebellum impairs protein N-glycosylation and motor function. (A) Far-WB with SNA lectin in P7 cerebellum and (B) quantification (n = 4 per genotype), p-value=0.29. (C) WB analysis of LAMP1 expression in mouse embryonic fibroblasts (MEFs). (D) WB analysis of LAMP1 level from P7 cerebellum and quantification. * indicates a PNGase sensitive LAMP1 isoform; **p-value=0.0032. (E) WB analysis as described in (D) but with increased electrophoretic migration and adjusted protein amounts to highlight LAMP1 hypoglycosylation in the mutant sample. (G) Box plot of the latency to fall during rotarod testing (n = 15 for each condition). One-way ANOVA was used for rotarod statistics. For all others, two-tailed student t-test was used. **p<0.01; ***p<0.001. Results are presented as mean ± s.d.

DOI: https://doi.org/10.7554/eLife.38309.002

The following figure supplement is available for figure 1:

**Figure supplement 1.** Generation of conditional Srd5a3 knockout mice and extended biochemical and behavioral analyses.

DOI: https://doi.org/10.7554/eLife.38309.003
Srd5a3<sup>ﬂ/−</sup> and control mice (Figure 1—figure supplement 1H). The latency to reach the platform was significantly lower on the second trial than on the first one for control mice, but not for En1-Cre; Srd5a3<sup>ﬂ/−</sup> mice (Figure 1—figure supplement 1I). This result suggests a mild impairment of working memory in the mutant mice. In contrast, En1-Cre; Srd5a3<sup>3R/−</sup> mice showed a severe and highly significant (p=0.0007) motor coordination defect assessed with the rotarod test (Figure 1G). These results suggest that in the absence of Srd5a3, the impaired cerebellum function arises from a mild and potentially selective hypoglycosylation of glycoproteins.

**Conditional deletion of Srd5a3 disrupts cerebellum granule cell development**

Then, we wanted to examine cerebellar development after deletion of Srd5a3 by conducting a morphological and histological survey at P14, P21, 2 and 6 months. We observed a significant hypoplasia in the En1-Cre; Srd5a3<sup>ﬂ/−</sup> mice (Figure 2A–D). Examining the mutant cerebellum cytoarchitecture revealed an accumulation of scattered ectopic cell clusters in the external part of the molecular layer (ML; Figure 2E). These clusters were NeuN-positive, post-mitotic granule cells (GCs) (Figure 2F) that failed to migrate to the internal granule cell layer (IGL). These cells remained in the external granule cell layer (EGL), a transient germinal zone. We observed this pattern at all our investigated latter timepoints with a higher incidence of ectopic clusters in the posterior lobules (Figure 2—figure supplement 1). Our examination of the two major cerebellar cell populations, Purkinje cells (PCs) and Bergman glia (BG), did not reveal major cellular defects (Figure 2—figure supplement 2A–C). Cerebellar development relies on key glycosylated proteins expressed and secreted by PC or BG cells, such as Shh (Dahmane and Ruiz i Altaba, 1999) or dystroglycan (Nguyen et al., 2013). So, we investigated the origin of the identified GCs ectopias. We deleted Srd5a3 using a GC-specific Cre line (Atoh1-Cre)(Matei et al., 2005) (Figure 2—figure supplement 2D). Atoh1-Cre; Srd5a3<sup>3R/−</sup> mice exhibit similar GC ectopias although in a lesser extent compared to the En1-Cre; Srd5a3<sup>3R/−</sup> mice (Figure 2G, Figure 2—figure supplement 1C,D). This observation indicates a partial GC-autonomous defect and could be explained by the involvement of GC glycoproteins and non-GC interactors that interplay to allow GCs to initiate their migration and prevent the formation of these ectopic clusters.

Taken together, our results support a critical role for Srd5a3 during cerebellar granule cells development likely through a GC-autonomous mechanism involving the glycosylation of specific, but undetermined, proteins.

**Cerebellum-specific Srd5a3 deletion decreases the levels of highly glycosylated proteins**

Next, we evaluated the molecular mechanisms underlying our observed cerebellar defect by conducting a total proteomic analysis on the developing P7 mouse cerebellum (Lipecka et al., 2016; Wiśniewski et al., 2009). This approach quantified 1982 proteins, whose abundance profiles can cluster each sample by genotype (Figure 3—figure supplement 1A, Supplementary file 1). Our statistical analysis identified 97 differentially abundant proteins (DAP) (~ 5% of the total; q-value <0.05) in the En1-Cre; Srd5a3<sup>3R/−</sup> mice (Figure 3A). To determine the deregulated pathways among the highly active, neural development pathways at this stage, we performed an over-representation analysis of the 97 DAP using ConsensusPathDB. Our analysis indicated that different pathways involved in ion and amino acid transport, synapse function, cell adhesion-related signaling and cholesterol biosynthesis interplay to generate the mouse phenotype (Figure 3B). Aside from changes in cholesterol metabolism, all enriched pathways contained at least one deregulated N-glycoprotein. Protein N-glycosylation is critical for ER-protein folding, however we did not find any enrichment in the ER stress pathway. Manual inspection of the 97 DAP revealed increased levels of only two ER-stress-related proteins, SDF2L1 (Fujimori et al., 2017) and HYOU1 (Zhao et al., 2010), while BiP, the classical ER-stress marker(Ron and Walter, 2007), showed a 1.3 fold non-significant (q = 0.08) increase (Supplementary file 1). We then performed a transcriptomic study on additional samples at the same stage, which confirmed the absence of any significant deregulation of the most widely used UPR markers (Figure 3—figure supplement 1B). In addition, we did not observe any change in BiP levels restricted to ectopic GC clusters (Figure 3—figure supplement 1C,D). Surprisingly, our results exclude a main effect of deregulated N-glycosylation processes on ER stress.
Figure 2. Reduced cerebellum size and ectopic granule neurons in the Srd5a3 mutant. (A) Dorsal view of P21 posterior brains, scale bar 2 mm. (B) Quantification of the cerebellar anterior-posterior and medio-lateral axes (n = 4 for each genotype), **p-value=0.0018. (C) Representative images of hematoxylin-eosin (HE) stained P21 sagittal slices of control and mutant cerebella, scale bar 1 mm. Lobule numbers are indicated. (D) Quantification of the midline sagittal cerebellar area (Control, n = 5; En1-Cre; Srd5a3fl/−, n = 4); *p-value=0.0245. (E) Magnification of HE-stained cerebellar cortex, scale bar 200 μm. All examined mutants show ectopic cell clusters (arrow-head) in the outer part of the molecular layer (ML). (F) DAPI (blue) and NeuN (red) staining of P21 En1-Cre; Srd5a3fl/− cerebellum. Ectopic cells (arrow-head) are positive for the post-mitotic GCs marker NeuN, scale bar 50 μm. (G) Representative image of an HE-stained sagittal slice of Atoh1-Cre; Srd5a3fl/− cerebellum showing similar ectopic cells in the outer ML under GC-specific Srd5a3 deletion, scale bar 1 mm and 100 μm, respectively (n = 4). Two-tailed Student t-test was used for statistics. *p<0.05, **p<0.01. Results are presented as mean ± s.d.

DOI: https://doi.org/10.7554/eLife.38309.004

The following figure supplements are available for figure 2:

Figure supplement 1. Cerebellar granule cell ectopias quantification.
DOI: https://doi.org/10.7554/eLife.38309.005

Figure 2 continued on next page
One possible consequence of defective protein N-glycosylation is decreased stability of hypoglycosylated proteins. We observed a significant, 4-fold enrichment of N-glycoproteins among the DAP with decreased levels (simplified as decreased N-glycoproteins; p=0.0012, Figure 3A). Examining this group of decreased N-glycoproteins revealed that they have more N-glycosylation sites (i.e. higher site multiplicity) with an average of 5.57 sites versus 3.24 in unchanged N-glycoproteins.

### Figure 3.
Disrupted neural pathways in the Srd5a3 mutant developing cerebellum. (A, upper panel) Pie chart representing the total proteomics analysis in P7 cerebellum (n = 4 for each genotype). 97 proteins were detected as differentially abundant proteins (DAP) in the mutant samples (q-value < 0.05). (A, lower panel) Distribution of unchanged proteins (white box), increased DAP (green box) and decreased DAP (red box) into ER stress-related (GO:0034976) or N-glycoproteins (Reference glycoproteomic database, see Materials and method) categories. A significant enrichment was detected within the decreased DAP for N-glycoproteins (n = 8, Fisher exact test, p-value=0.0012). (B) Over-representation analysis on DAP performed with ConsensusPathDB using the 1982 detected proteins as background. All pathways with p-value<0.01 are represented. N-glycoproteins are indicated in green. (C) Average multiplicity in the unchanged and the decreased N-glycoproteins groups. (D) Enrichment for different glycoprotein categories among the decreased N-glycoproteins. Multiplicity represents groups of proteins with more than one (≥2), two (≥3) or three (≥4) N-glycosylation sites. Multiplicity of ≥1 represents all of the decreased N-glycoproteins and is set to one (no enrichment). There is a significant 2.4 fold-change enrichment for glycoproteins with four or more N-glycans among the decreased N-glycoproteins (Fischer exact test, p-value=0.0378).

DOI: https://doi.org/10.7554/eLife.38309.007

The following figure supplement is available for figure 3:

**Figure supplement 1.** Extended total proteomic analysis of the unfolded protein response (UPR).

DOI: https://doi.org/10.7554/eLife.38309.008
Indeed, N-glycoproteins with four or more N-glycosylation sites are significantly enriched within the decreased N-glycoproteins (Figure 3D). These data demonstrate that the mild glycosylation defect detected in the Srd5a3 mutant cerebellum disrupts the levels of highly glycosylated proteins, but does not disrupt ER homeostasis.

### N-glycosylation site multiplicity and primary sequence underlies the selective protein glycosylation defect

To further elucidate the N-glycosylation deficiency, we included a lectin-affinity based (ConA, WGA, RCA120) enrichment step at the peptide level, followed by deglycosylation (Zielinska et al., 2010), prior proteomic analysis (Figure 4—figure supplement 1A, data are available via ProteomeXchange with identifier PXD009906). This enrichment can identify N-glycosylation sites and quantify the abundance of each site when they are glycosylated. Using this dataset, we identified 140 likely new glycosylation sites (≈8% of the total, see Materials and method, Supplementary file 2) with high recurrence of non-canonical sequons (≈40% of the new sites, Supplementary file 2). Most proteins carrying these sites are intrinsic to membrane as observed for proteins with previously identified glycosylation sites (Gene Ontology analysis in Supplementary file 2). In total, we identified 1404 glycopeptides detected in at least 3 out of 4 control samples (Figure 4A, Supplementary file 2). As we observed for the total proteome, we could cluster the samples according to the genotype based on the expression levels of all the glycopeptides (see Materials and method, Figure 4—figure supplement 1B). Total proteomics and glycoproteomic analysis yielded complementary information. Total proteomics provides information of the protein abundance level but can only detect peptides containing an unoccupied N-glycosylation site. Glycoproteomics only allows detection of glycopeptides if the site is occupied. For 15% of the N-glycopeptides detected in glycoproteomics (n = 206), we found decreased levels in the mutant samples. Of these, we only detected 24 N-glycopeptides in the previous total proteomic experimental settings, consistent with the high enrichment observed with the glycoproteomics analysis (Figure 4—figure supplement 1C, Supplementary file 3). Among these peptides, 13 were never detected in control but only in mutant samples in total proteomics. These data indicate that these 13 peptides were not glycosylated in the mutant. We conclude that most differences we detected in the glycoproteomic experiment reflect reduced glycosylation site occupancy.

We also detected an extremely significant correlation between N-glycan multiplicity and N-glycopeptide levels (Figure 4B, Mann-Whitney test, p-value<0.0001). This result is consistent with our larger glycoprotein dataset. To delineate the origin of reduced occupancy of individual N-glycosylation sites, we analyzed their primary sequences (Figure 4C). We identified a mild effect of the presence of a non-aromatic amino-acid (other than Phe, Tyr, His or Trp) at position –2 and a more significant effect of the Serine at position +2 (Figure 4D), as previously described (Gavel and Heijne, 1990; Murray et al., 2015), with an increased impact in highly glycosylated proteins (Figure 4E). These findings demonstrate that N-glycan multiplicity combined with sequon efficiency is a major parameter driving protein sensitivity to N-glycosylation defects.

### Impaired protein N-glycosylation targets IgSF-CAMs

We next wanted to delineate the affected pathways based on our glycoproteomic results. We performed an over-representation analysis using proteins carrying at least one N-glycopeptide significantly decreased in mutant samples (145 N-glycoproteins, 206 N-glycopeptides). This analysis revealed that the most significantly affected proteins (4 out of the top 10 deregulated pathways) derive directly from cell adhesion and axon guidance-related pathways involving adhesion proteins from the L1CAM family (Figure 5A, red circles). L1CAMs and the previously identified NCAM1 andCNTN2 (Figure 3B) belong to the immunoglobulin superfamily of cell adhesion molecules (IgSF-CAMs) with critical roles in brain development (Maness and Schachner, 2007; Pollerberg et al., 2013; Stoeckli, 2010). We speculate that this particular sensitivity of IgSF-CAMs to a N-glycosylation defect arises from their higher N-glycosylation site multiplicity with an average of 3.3 sites versus 2.3 in non IgSF-CAM proteins (Calculated based on the reference glycoproteomic dataset, Supplementary file 2, Mann Whitney test, p-value<0.0001).

So, we next hypothesized that impaired IgSF-CAMs function derived from sub-optimal N-glycosylation and that defective IgSF-CAMs contributed to our observed histological defect.
We confirmed the enrichment for IgSF-CAMs found in our over-representation analysis at the N-glycopeptide level (18.4% of the decreased N-glycopeptides belong to an IgSF-CAM protein, Fisher exact test, p-value=0.0058, Figure 5B).

We selected two highly N-glycosylated IgSF-CAMs relevant for the granule cell histological phenotype for validation by WB. We did observe a clear hypoglycosylation defect for L1CAM and...
**Figure 5.** IgSF-CAMs are especially sensitive to a mild N-glycosylation impairment. (A) Graphical representation of enriched pathways and their interactions within proteins with reduced N-glycopeptide in the mutant (ConsensusPathDB). The nodes size indicates the number of proteins within the pathway, and their shade notes the significance (the darkest, the more significantly enriched). Red circles enclose the L1CAM family-related pathways. (B) Volcano plot against all N-glycopeptides. The decreased (red) or increased (blue) N-glycopeptides belonging to an IgSF-CAM protein are highlighted. There is enrichment for IgSF-CAMs glycopeptides within the decreased ones (Fisher exact test, p-value=0.0058). (C) WB against L1CAM and NrCAM in the P7 mouse cerebellum and (D) quantification of the 70 kDa cleaved isoform of L1CAM (n = 3 for each genotype, p-value=0.0312). (E) Multiplicity per protein of three IgSF-CAMs subfamilies (L1CAMs, CNTNs and NCAMs) in different species. Multiplicity information was collected from experimental datasets (Zielinska et al., 2010) and (Zielinska et al., 2012) except for human information extracted from uniprot database. (F) WB analysis of L1CAM and NrCAM levels in CPNs (n = 2 per genotype, repeated twice). Unless indicated, two-tailed Student t-test was used for statistics. Results are presented as mean ± s.d. *p<0.05.

DOI: https://doi.org/10.7554/eLife.38309.011

The following figure supplements are available for figure 5:

**Figure supplement 1.** Schematic representation of the mouse L1CAM and NrCAM with their hypoglycosylated sites.

DOI: https://doi.org/10.7554/eLife.38309.012

**Figure supplement 2.** hiPSCs differentiation towards human cortical projection neurons (CPNs).

DOI: https://doi.org/10.7554/eLife.38309.013
Neuroscience

L1CAM and NrCAM, respectively, in these IgSF-CAMs along the axons and at the growth cone but with 30.8% and 39.6% decrease for domain in non-permeabilized cells (see Materials and methods). We detected the expression of L1CAM and NrCAM using antibodies directed against their ecto-

In order to evaluate the consequences of IgSF-CAM hypoglycosylation at the cellular level, we stud-

Hypoglycosylation of L1CAM and NrCAM alters their expression level at the plasma membrane

In order to evaluate the consequences of IgSF-CAM hypoglycosylation at the cellular level, we studied the cell surface expression of L1CAM and NrCAM using antibodies directed against their ecto-domain in non-permeabilized cells (see Materials and methods). We detected the expression of these IgSF-CAMs along the axons and at the growth cone but with 30.8% and 39.6% decrease for L1CAM and NrCAM, respectively, in Srd5a3 cKO neurons (Figure 6A,B). We recapitulated a similar defect using Tunicamycin suggesting that the disruption of other steps of the N-glycosylation pathway as well as specific drugs can efficiently alter the cell-surface expression of these glycoproteins. Such differences in the membrane expression levels are expected to have consequences on the ability of neurons to bind their substrates. Both L1CAM and NrCAM are known to interact homophilically and heterophilically with multiple IgSF-CAM, e. g. CNTN1 and CNTN2 (Falk et al., 2002; Sonderegger, 1997). We decided to test for the homophilic binding activity, by incubating cultured GCs with chimeras made of the extracellular part of L1CAM or NrCAM fused with the IgG Fc region (L1CAM-Fc and NrCAM-Fc, respectively). Indeed, a 38.5% and 56.0% decrease in the binding of L1CAM and NrCAM chimeras, respectively, was observed in mutant cells (Figure 6C,D). This result validates the functional impact of IgSF-CAMs hypoglycosylation and suggests that the glycosylation level of IgSF-CAMs plays a critical role in the regulation of their cell-surface expression.

Srd5a3 is necessary for IgSF-CAM-dependent cerebellar granule cells adhesion and axon guidance

IgSF-CAMs rely on their glycan charge to interact with each other (Wei and Ryu, 2012) (Horstkorte et al., 1993). Proper IgSF-CAMs trans-interaction is essential for adequate nervous system connectivity, for fasciculation and axonal guidance(Pollerberg et al., 2013). To demonstrate that IgSF-CAMs hypoglycosylation is directly involved in En1-Cre; Srd5a3fl/fl mouse phenotype, we examined the neurite dynamics by live cell imaging of isolated GCs cultured under different surface coating conditions. En1-Cre; Srd5a3fl/fl GCs did not show any significant alterations that suggested differences in proliferation or cell death onto any substrates (Figure 7—figure supplement 1A). In contrast, GCs showed defective neurite sprouting with a 20% decrease when using a coating with laminin and poly-D-lysine (Figure 7A,B). This phenotype was exacerbated in the presence of coatings made of human recombinant L1CAM or NrCAM proteins that enhance homophilic interactions (Dequidt et al., 2007). We observed a 37% and 52% reduction in neurite number for mutant GCs compared to control, on L1CAM and NrCAM substrates, respectively (Figure 7C). A significant decrease was also observed in neurite length (Figure 7—figure supplement 1B) but less specifically associated with IgSF-CAM coating (Figure 7—figure supplement 1B–D). To further link the IgSF-CAM-dependent neurite development in the mutant GCs to the defective initiation of cell migration, we used GC re-aggregates to evaluate cellular migration in vitro under different coating conditions (Bizzoca et al., 2003) (Katic et al., 2014; Kerjan et al., 2005). Using laminin and poly-D-lysine coating, the mutant re-aggregates showed no significant difference in neurite number and only a mild...
**Figure 6.** L1CAM and NrCAM hypoglycosylation affects their cell surface expression and binding activity. (A) L1CAM and (B) NrCAM surface immunostaining of cultured GCs and quantification. (C) L1CAM-Fc and (D) NrCAM-Fc binding assay on cultured GCs. The cells were incubated with the recombinant proteins and bound L1CAM-Fc or NrCAM-Fc were visualized with anti-Fc antibody. All of the staining and quantifications were performed with at least three biological replicates from different litters. Each dot represents a single neuron. Scale bar 10 μm. Results are presented as mean (black line)±s.d.

DOI: https://doi.org/10.7554/eLife.38309.014
**Figure 7.** IgSF-CAM-dependent neurite dynamic and axon orientation defects in Srd5a3 mutant cerebellum. (A) Neurite number per GC body cluster across 21 hr in laminin and poly-D-lysine-coated wells as measured with Incucyte live cell imaging system (Control, n = 3; En1-Cre; Srd5sa3<sup>fl/-</sup>, n = 3). (B) Representative image of control and En1-Cre; Srd5sa3<sup>fl/-</sup> GCs after 21 hr in laminin and poly-D-lysine-coated wells. Scale bar 100 µm. (C) Neurite number normalized to GC body clusters after 21 hr with laminin/poly-D-lysine coatings, fibronectin or IgSF-CAMs-coated wells (NrCAM and L1CAM). Each dot
per coating represents results from a single mutant mouse. All GC cultures were performed in technical triplicates (Control, n = 3; En1-Cre; Srd5a3fl/fl, n = 3). (D) Neurite number and number of migrating neurons in GC re-aggregates plated for 24 hr on laminin and poly-D-lysine and (E) L1CAM coated surfaces. Five aggregates were quantified per mouse and coating condition (Control n = 5, En1-Cre; Srd5a3fl/fl, n = 7). (F) Representative electron microscopy images of cerebellar ML sagittal view at P21 taken from control (n = 3) and Srd5a3 mutant (En1-Cre; Srd5a3fl/fl, n = 1; Atoh1-Cre; Srd5a3fl/fl, n = 2) mice. Scale bar 2 μm. Parallel fibers show a single orientation in control ML, whereas some exhibit an abnormal perpendicular orientation in the most outer ML in the mutant mice (G, red arrowhead). Two-tailed Student t-test was used for statistics. *p<0.05; **p<0.01. Results are presented as mean ± s.d.

DOI: https://doi.org/10.7554/eLife.38309.016

The following figure supplement is available for figure 7:

Figure supplement 1. Changes in granule cell (GC) number and neurite length under different coating conditions.
DOI: https://doi.org/10.7554/eLife.38309.016

---

**Discussion**

We sought to delineate the impact of Srd5a3-driven hypoglycosylation of N-glycoproteins during development, a biochemical defect likely shared between multiple CDG. Here, we generated a conditional KO mouse that recapitulates some neurological symptoms induced by this ER glycosylation defect. We hypothesized that patients with SRD5A3-related and other types of CDG exhibit protein hypoglycosylation leading to cerebellar dysfunction. From a physiological perspective, we hypothesized that the characterization of the affected glycoproteins and glycosylation sites would identify new roles for protein N-glycosylation during central nervous system development. So, we combined total proteome and glycoproteome approaches to identify the molecular mechanisms underlying the observed cerebellar dysfunction and GC histological defects. Our strategy assessed the relative occupancy at each N-glycosylation site regardless of the N-glycan nature. The pleiotropic roles of glycans on development and synaptic transmission suggest that genetic defects in the synthesis of the N-glycan core structure would have multiple distinct and integrated consequences. We unexpectedly found that partial inhibition in N-glycosylation during cerebellar development has persistent consequences on a specific set of glycoproteins and pathways. Among them, we found that IgSF-CAMs-mediated cell adhesion is the most consistently affected. Our further investigations revealed impaired GC adhesion and axon growth, which support IgSF-CAM hypoglycosylation as the primary underlying defect. Our glycoproteomic and total proteomic analyses strongly suggest an intuitive mechanism where N-glycan multiplicity is a critical factor driving the molecular defects.

SRD5A3 acts at the last step of dolichol synthesis and facilitates also the synthesis of O-mannose-linked glycans, C-mannosylation, and glycoporphosphatidylinositol (GPI) anchored proteins. However, N-glycosylation is the most demanding cellular process with eight dolichol molecules needed for each N-linked glycan(Cantagrel and Lefeber, 2011). Defects in SRD5A3 alter lipid membrane composition that subsequently impairs protein N-glycosylation. Consistently with previous results using Srd5a3 gene-trapped embryos(Cantagrel et al., 2010), several enzymes from the cholesterol/mevalonate pathway were found deregulated (Figure 3B). These observations indicate a regulatory mechanism that could alleviate these glycosylation defects by stimulating the mevalonate pathway (Haeuptle et al., 2011) (Welti, 2013). While we cannot exclude defects in other glycosylation
pathways, our results demonstrate that changes in N-glycosylated proteins and correlations between the N-glycan number and peptides abundance in both proteomic and glycoproteomic analyses strongly implicate the N-glycosylation pathway as the major metabolic target underlying the phenotype and neurological symptoms in SRD5A3-CDG. Surprisingly, we did not observe a signature of the UPR pathway as seen in other CDG models. This result does not exclude a minimal glycoprotein folding impairment that does not significantly alter the ER homeostasis. A potential mechanism of adaptation to this mild impairment is detected with the over-expression of the HYOU1 and SDF2L1 chaperones.

Our work shows that proteins with high N-glycan multiplicity, estimated to four or more sites, show an increased risk for hypoglycosylation to induce protein degradation or dysfunction. Moreover, differences in the sequon can have an additive effect to this increased risk. Although several highly-glycosylated proteins are frequently used as sensitive biomarker for N-glycosylation defects (He et al., 2012) (Park et al., 2014), we provide initial evidence that the total number of glycosites per protein is a major parameter that influences N-glycoproteins sensitivity. Our total proteomic analysis clearly indicates that decreased glycoprotein abundance is a direct consequence of incomplete glycosylation of highly glycosylated proteins. Yet, examining individual proteins, such as the IgSF-CAM member L1CAM, did not reveal significant differences in full-length isoform abundance by western blot but showed altered cleavage as well as reduced cell surface expression as a consequence of its hypoglycosylation. The cleaved L1CAM isoform (70 kDa) enhances cerebellar GC neurite outgrowth (Lutz et al., 2014), which indicates that hypoglycosylation of some proteins can impair their processing, trafficking and/or functioning without necessarily causing significant changes in their cellular abundance.

The number of N-glycans, in conjunction with their degree of branching, regulates cell proliferation and differentiation. Highly glycosylated growth factor receptors show increased cell-surface expression by forming galectin-mediated molecular lattices (Lau et al., 2007). We cannot exclude that some cell adhesion proteins utilizing a similar mechanism also show alterations in the Srd5a3 mutant cerebellum. To our knowledge, neuronal galectin-IgSF-CAM interactions remain unreported. The pivotal role of IgSF-CAMS in cell migration, axonal guidance and synaptogenesis has been widely studied in mouse (Sakurai et al., 2001) (Fransen et al., 1998) (Berglund et al., 1999) (Çakakoğlu et al., 2014) (Sytnyk et al., 2017). However, we still understand very poorly how N-glycans contribute to these functions with few exceptions such as the established role for the polysialic acid modification of NCAM1 N-glycan chains (Weinhold et al., 2005). Interestingly, specific N-glycosylated residues, alpha2,3-linked sialic acid and Lewis(x) of L1CAM, CNTN1 and CNTN2 have been reported to mediate interaction with the glycopeptide CD24 modulating cell adhesion and neurite outgrowth of cerebellar neurons (Lieberoth et al., 2009).

Among proteins identified in our proteomic approaches, NrCAM and L1CAM show redundant functions in cerebellar GC development, in addition to their roles in axonal growth (Sakurai et al., 2001) (Demyanenko et al., 1999) (Demyanenko et al., 2011). CNTN2/TAG-1 contributes to neuronal polarization and axonal organization. Interestingly, the corresponding KO mouse shows ectopic GC clusters at the pial surface of the cerebellum (Xenaki et al., 2011), as observed in the Srd5a3 mutant cerebellum. Mice lacking Cntr1 show aberrant parallel fibers distribution and motor coordination defects (Berglund et al., 1999). Since neural IgSF-CAMS largely bind to each other homophilically and heterophilically (Pollerberg et al., 2013), we suspect that the observed neuronal adhesion defects arise from a cumulative effect of partial hypoglycosylation of multiple interacting partners (Schwarz et al., 2009) (Stoeckli et al., 1997). IgSF-CAMS show increased complexity and members in vertebrates (Vogel and Chothia, 2006). The increased diversity in some IgSF-CAM proteins correlates with the presence of homologs with higher multiplicity in mammals (Figure 5E). We speculate that a positive selection of adhesion proteins with high N-glycosylation site multiplicity can support the increased complexity of mammalian neuronal organization and connectivity.

In addition to the central nervous system, highly glycosylated IgSF-CAM members also play critical roles in other systems affected in CDG patients, such as the developing eyes (Morava et al., 2009) and immune system with ICAM1 (Blank et al., 2006; He et al., 2014), or in other diseases like cancer, where L1CAM plays a critical role in tumor metastasis (Kiefel et al., 2012) (Agrawal et al., 2017). Further investigations will determine how IgSF-CAM hypoglycosylation regulates or alters the clinical outcomes and responsibility to therapeutic strategies. In conclusion, our study demonstrates that suboptimal functioning of the ER-glycosylation machinery impairs primarily highly glycosylated
N-glycoproteins with mild or no effect on ER homeostasis. We propose that the prevalent neurological symptoms observed in SRD5A3-CDG patients and likely other types of CDG type I result from defective neural cell adhesion, caused by IgSF-CAM hypoglycosylation. Deciphering how multiple N-glycans can influence differential IgSF-CAM adhesion properties will address important unresolved neurobiological questions regarding neuronal migration, axonal outgrowth and synaptogenesis.

### Materials and methods

#### Key resources table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Genetic reagent (M. musculus)     | ACTB:FLPe B6J | JAX stock #005703   | ACTB:FLPe B6J |                         |
| Genetic reagent (M. musculus)     | Atoh1-Cre   | JAX stock #011104   | Tg(Atoh1-cre)1Bfri |                      |
| Genetic reagent (M. musculus)     | CAG-Cre     | JAX stock #004682   | B6.Cg-Tg (CAG-cre/Esr1*)JAmc/J |           |
| Genetic reagent (M. musculus)     | CMV-Cre     | PMID: 7624356       | En1tm2(cre)Wrst |                   |
| Genetic reagent (M. musculus)     | En1-Cre     | JAX stock #007916   | En1tm2(cre)Wrst |                   |
| Genetic reagent (M. musculus)     | R26RLacZ    | PMID: 25594525     |             |                   |
| Genetic reagent (M. musculus)     | Srd5a3Floxed | IMPC - ES cell clone EPD0575_2_F01 carrying the Srd5a3tm1a (EUCOMM)Wtsi allele | Srd5a3tm1a (EUCOMM)Wtsi |     |
| Antibody                          | BiP         | Enzo                | ADI-SPA-826 | 1:50                   |
| Antibody                          | Calbindin   | Swant               | CB-38A      | 1:10,000               |
| Antibody                          | GFAP        | Millipore           | AB5804; RRID: AB_2109645 | 1:1,000 |
| Antibody                          | L1CAM       | Abcam               | AB24345; RRID: AB_448025 | 1:2,000 |
| Antibody                          | L1CAM       | Appel et al. (1995) | S57.B6; Membrane staining |     |
| Antibody                          | Laminin     | Sigma               | L9393; RRID: AB_477163 | 1:33 |
| Antibody                          | LAMP1       | Abcam               | AB24170; RRID: AB_775978 | 1:2,000 |
| Antibody                          | NeuN        | Millipore           | MB377; RRID: AB_2298772 | 1:200 |
| Antibody                          | NrCAM       | Abcam               | AB24344; RRID: AB_448024 | 1:2,000/Also used for membrane staining |
| Antibody                          | Tuj1        | Sigma               | T2200       | 1:2,000               |
| Antibody                          | β-actin     | Thermo Fisher       | AM4302; RRID: AB_2536382 | 1:20,000 |
| Chemical compound, drug           | D-Lysine    | Sigma               | P6407       |                         |
| Chemical compound, drug           | Fibronectin | Sigma               | F2006       |                         |
| Chemical compound, drug           | Laminin     | Sigma               | 11243217001 | Coating reagent        |
| Chemical compound, drug           | Lectin ConA | Sigma               | C2272       | 1:2,000               |

*Continued on next page*
### Animal care

Animals were used in compliance with the French Animal Care and Use Committee from the Paris Descartes University (APAFIS#961 – 201506231137361). All behavioural analysis were performed in compliance with the guidelines of the French Ministry of Agriculture for experiments with laboratory animals. The experimental protocol and euthanasia have been approved by the Ethical Committee 27, registered at the French ministry of research.

### Generation of the Srd5a3 conditional KO mouse

Embryonic stem (ES) cell clone EPD0575_2_F01 carrying the Srd5a3<sup>tm1a(EUCOMM)Wtsi</sup> allele was acquired from the International Mouse Phenotyping Consortium (IMPC; http://www.mousephenotype.org/). After additional QC validation based on Southern-blot, ES cells were injected into C57BL/6 at the University of California, San Diego Transgenic and Gene targeting Core yielding chimeric mice. Chimera were bred to C57BL/6 until germline transmission was successful to produce knockout first allele. Then, the Srd5a3<sup>tm1a</sup> mice were bred with ACTB:FLPe B6J (JAX stock #005703) Flp recombinase expressing transgenic mice to produce mice expressing a functional Srd5a3 allele that retained flox sites surrounding exon 2 to 4 (out of the six exons) and including part of the enzymatic domain (PFAM, Steroid_dh). After outbreeding the Flp recombinase transgene, Srd5a3 floxed mice were crossed with hemizygous CMV-Cre line transgenic mice (Metzger et al., 1995) to generate Srd5a3 KO allele. Srd5a3 floxed mice were bred with hemizygous En1<sup>tm2(cre)Wrsi</sup> transgenic mice (JAX stock #007916) expressing Engrailed1 promoter driven Cre to produce cerebellum-specific deletion (En1Cre; Srd5a3<sup>fl/-</sup>) or with hemizygous Tg(Atoh1-cre)1Bfri (JAX stock #011104) expressing Atoh1 promoter driven Cre to produce cerebellum granule cell-specific deletion (Atoh1Cre; Srd5a3<sup>fl/-</sup>). Specific Cre expression was confirmed by breeding with a LacZ

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|-------------------|------------|-----------------------|
| Chemical compound, drug          | Lectin ConA | Sigma             | C2010      | Glycoproteomics reagent |
| Chemical compound, drug          | Lectin RCA120 | CliniSciences    | L-1080     | Glycoproteomics reagent |
| Chemical compound, drug          | Lectin SNA  | Vector laboratories | B-1305     | 1:2.000               |
| Chemical compound, drug          | Lectin WGA  | Sigma             | L9640      | Glycoproteomics reagent |
| Chemical compound, drug          | PNGase      | New England BioLabs | P0704L     |                       |
| Chemical compound, drug          | Tamoxifen   | Sigma             | H6278      | 1 μM                  |
| Chemical compound, drug          | Tunicamycin | BioTechne         | 3516/10    | 0.01 μg/mL            |
| Chemical compound, drug          | WATER-18O, 97 ATOM % 18O | Sigma | 329878    | Glycoproteomics reagent |
| Commercial assay or kit          | GeneChip Mouse Transcriptome Array 1.0 | Affimetrix | MTA1.0         |                       |
| Peptide, recombinant protein     | L1CAM-Fc    | R and D           | 777-NC     |                       |
| Peptide, recombinant protein     | NrCAM-Fc    | R and D           | 2034-NR    |                       |
| Software, algorithm              | ParTek Genomics Suite | ParTek |           |                       |
| Other                             | IncuCyte    | Essen Bioscience | IncuCyte S2 with NeuroTrack module |
reporter-carrier mouse line (R26R^{LacZ} mouse). Efficient Srd5a3 recombination was assessed by RT-PCR and RT-qPCR in the absence of any specific antibody.

**Mouse embryonic fibroblasts (MEFs) generation**

For MEFs generation, Srd5a3^{-/-} mice were bred with CAGCre mice (B6.Cg-Tg(CAG-cre/Esr1*)5Amc/J; CAGCre; Srd5a3^{-/-}). CAGCre;Srd5a3^{-/-} embryonic fibroblasts were isolated at E14.5 and immortalized by serial passaging as described by Xu, 2005. For Srd5a3 recombination primary MEFs were treated for 4 days with tamoxifen (1 μM, H6278, Sigma) prior to immortalization. Efficient Srd5a3 recombination was assessed by RT-qPCR after immortalization.

**Behavioral studies**

For behavioral analysis, 15 En1Cre; Srd5a3^{-/-} and 15 control littermates, gender-matched, 2 – 3 months old were used. The number of animals per group was chosen as the optimal number likely required for conclusion of statistical significance, established from prior experience using the same behavioral tests (Pereira et al., 2009). Morris water maze (MWM) test was used to assess working memory. Prior to the test, the mouse swimming speed was analyzed. No differences in the swimming speed were detected. For MWM, mice were exposed twice to the same hidden platform for a total of eight sessions. The average latency for each mouse is represented. The improvement to find the platform on the second trial was evaluated. For motor coordination, classic accelerating rotarod testing was done for 10 min and for a total of three sessions, 24 hr apart. The first three falls from the rod during each session were annotated. A statistical Grubbs test was performed to verify the absence of significant outliers in each group.

**Histological analysis**

Gross anatomical analyses and HE staining were performed as previously described (Akizu et al., 2013). The resulting slices were scanned with NanoZoomer-XR (Hamamatsu Photonics, Japan). We evaluated the cerebellum size difference by measuring the vermis area from mid-sagittal sections (Pogoriler et al., 2006). We identified the midline by the absence of fastigial nucleus and cerebellar peduncles. A single midline section was used to calculate the area with ImageJ. For quantification of the surface occupied by the clusters and their distribution, two mice and one slice per region were quantified per genotype. For IHC, the cerebellum was embedded in OCT. 12 μm-thick slices were generated with cryostat. The following antibodies were used for IHC: NeuN (1:200, MAB377; Millipore), Calbindin (1:10.000; CB-38A, Swant), GFAP (1:1.000; AB5804, Millipore), laminin (1:33, L9393, Sigma) and BiP (1:50, ADI-SPA-826, Enzo). All samples were mounted with ProLong Gold Antifade Mountant with DAPI (Life Tech). Images were taken with confocal Leica SP8 STED and analyzed with ImageJ.

**Protein extraction and western blotting**

P7 mouse cerebellum samples and MEFs were isolated in RIPA buffer (1% SDS; 0,1% for cell extracts) supplemented with EDTA-free protease inhibitor (1183617001, Sigma) and phosphatase inhibitor cocktail (4906845001, Sigma), homogenized by sonication (Bioruptor Pico sonication device – 8 cycles 30”ON/30”OFF) and centrifuged at 12.000 g at 4°C for 20 min. The supernatant was recovered and quantified with BCA (Life technologies). For cell extracts, RIPA was added directly to the flask and the cells were recovered with a cell scrapper followed by the same protocol. Equal amounts of protein were loaded from each sample in polyacrylamide gels. Gel transfer to nitrocellulose membranes was performed with the Trans-Blot Turbo Transfer System for 10 min at 1.3A and 25V. Membranes were blocked with 5% dry milk and incubated O/N at 4°C with the following antibodies: LAMP1 (1:2.000; AB24170, Abcam), L1CAM (1:2.000; AB24345, Abcam), NrCAM (1:2.000; AB24344, Abcam) and β-actin (1:20.000; AM4302, Thermo Fisher). Depending on the antibody suitability, the membrane was developed by HRP system (LAMP1, ChemiDoc XRS + System) or with fluorescent secondary antibodies (Odyssey CLx imaging system). All secondary antibodies were used at 1:10000. For far-western blotting, no blocking step was performed, and the membrane was directly incubated for 1 hr with biotinylated SNA (1:2.000; B-1305, Vector laboratories) or ConA (1:2000; C2272, Sigma) and posteriorly with IRDye 800CW-streptavidin for 1 extra hour (1:10.000;...
RNA extraction, RT-PCR, RT-qPCR and transcriptomic analysis

RNA was extracted with Trizol reagent (15,596–026, Thermo Fisher) according to manufacturer’s instructions. For transcriptomic studies, En1Cre;Srd5a3fl/fl P7 mice (n = 4) and control littermates (n = 4) were used. RNA quality was validated with Bioanalyzer 2100 (using Agilent RNA6000 nano chip kit) and 180 ng of total RNA were reverse transcribed using the GeneChip WT Plus Reagent Kit (Affymetrix). The resulting double strand cDNA was used for in vitro transcription with T7 RNA polymerase (WT cDNA synthesis and amplification kit, Affymetrix). After purification according to Affymetrix protocol, 5.5 µg of the cDNA obtained were fragmented and biotin-labelled using Terminal Transferase (WT terminal labelling kit, Affymetrix). cDNA was then hybridized to GeneChip Mouse Transcriptome Array 1.0 (MTA 1.0., Affymetrix) at 45°C for 17 hr. After O/N hybridization, chips were washed on the fluidic station FS450 following specific protocols (Affymetrix) and scanned using the GCS3000 7G. The scanned images were then analyzed with Expression Console software (Affymetrix) to obtain raw data (cel files) and metrics for Quality Controls. The observations of these metrics and the study of the distribution of raw data showed no outlier experiment. Robust multi-array average (RMA) normalization was obtained using R, and normalized data were subjected to statistical tests. For RT-PCR/RT-qPCR 1 µg of RNA was retrotranscribed into cDNA with SuperScript II reverse transcriptase (18064014, Thermo Fisher). qPCR was performed with PowerUp SYBR Green Master Mix (A25777, Thermo Fisher). The following primers were used: mβActin (F 5’-TACAGCTTCACCACCCACCAGC-3’; R 5’-AAGGAAGGTGAAAAAGAGC-3’) and mSrd5a3 (F 5’-CCGGGCTATTGGCTGGGTGG-3’ and R 5’-CTGTCTCAGTGCCTCTAGGAATGG-3’).

Total proteomics and glycoproteomics

P7 En1Cre;Srd5a3fl/fl (n = 4) and control (n = 4) littermates were used for total proteomics and the same number, but distinct mice, were used for glycoproteomics. The cerebellar protein extraction was performed as described above. For total proteomics, 100 µg of protein were processed by filter-aided sample preparation (FASP) protocol, as described previously (Lipecka et al., 2016). Briefly, samples were applied to 30 KDa MWCO centrifugal filter units (UFC503024, Amicon Ultra, Millipore) mixed with 200 µl of urea (UA) buffer (8M urea, 100 mM Tris-HCl pH 8.8) and centrifuged twice. The samples were incubated for 20 min in the dark with UA buffer containing 50 mM iodocetamide for alkylation. The filter units were subsequently washed twice with UA buffer and twice more with ABC buffer (50 mM ammonium bicarbonate). Peptide digestion was carried by incubation with trypsin (1:50) O/N at 37°C. The resulting peptides were collected by two washes with ABC buffer, vacuum dried and dissolved in 0.1% (v/v) trifluoroacetic acid with 10% acetonitrile.

Glycoproteome analysis was performed by FASP with an additional step of enrichment in N-glycopeptides by lectins, as described by M. Mann and colleagues (Zielinska et al., 2010). Briefly, 100 µg of trypsinized peptides were recovered in binding buffer (20 mM Tris/HCl pH 7.6, 1 mM MnCl₂, 1 mM CaCl₂, 0.5; NaCl) and incubated with a lectin mixture (90 µg ConA, 90 µg WGA and 71.5 µg RCA₁₂₀) for 1 hr. To elute the non-glycosylated peptides, not attached to the lectins, the samples were washed four times with binding buffer, and after with ABC solution in H₂O₁₈ (P0704L, New England BioLabs) for 3 hr at 37°C. The N-glycopeptides were recovered by washing twice with ABC. All centrifugation steps were performed at 14,000 g at RT.

LC-MS/MS analysis

For each run, estimated 0.5 µg were injected in a nanoRS-LC-Q Exactive PLUS (Dionex RSLC Ultimate 3000, Thermo Scientific, Waltham, MA, USA). Peptides were separated on a 50 cm reversed-phase liquid chromatographic column (Pepmap C18, Dionex). Chromatography solvents were (A) 0.1% formic acid in water and (B) 80% acetonitrile, 0.08% formic acid. Peptides were eluted from the column with a linear gradient of 120 min from 5% A to 80% B followed by 27 min of column re-equilibration in 5% A. Two blanks, each with two 25 min-linear gradient, were run between samples to prevent carryover. Peptides eluting from the column were analyzed by data dependent MS/MS, using top-10 acquisition method. Briefly, the instrument settings were as follows: resolution was set to 70,000 for...
MS scans and 17,500 for the data dependent MS/MS scans in order to increase speed. The MS AGC target was set to 3.10^{6} counts with 200 ms for the injection time, while MS/MS AGC target was set to 1.10^{5} with 120 ms for the injection time. The MS scan range was from 400 to 2000 m/z. Dynamic exclusion was set to 30 s. All analyses were performed in technical triplicate for each biological replicate.

**Total proteomics and glycoproteomics data analysis**

The MS files were processed with MaxQuant software version 1.5.8.3 and searched with Andromeda search engine against the mouse subset from the UniProtKB/Swiss-Prot complete proteome database (release 2016_06). Statistical analysis and logo extractions were performed using Perseus version 1.5.5.3. Different thresholds were applied to total proteomics and glycoproteomics analysis given that the intensity of several peptides in total proteomics is used for determining protein intensity, while a single peptide in glycoproteomics is analysed at the time. For total proteomics, only proteins detected in all eight samples (4 controls and four mutants) were retained for statistical analysis, avoiding all data imputation. For comparative glycoproteomics, we retained glycosites detected in at least 3 out of 4 control samples. Additionally, we selected proteins that were specifically detected in control or mutant samples by retaining proteins detected solely in at least 3 samples of one group. Both FDR and p-value (q-value <0.05, paired student t-test) was used for total proteomics, whereas the p-value (<0.05, unpaired student t-test) was used for N-glycopeptides. As a database for N-glycoproteins and number of N-glycosylation sites per protein (qualitative dataset, reference glycoproteomic dataset), the data obtained by glycoproteomics was used: any glycopeptide detected in at least two control samples was considered as potentially N-glycosylated (Supplementary File 2). Sites that were not previously described in Zielinska et al. or not predicted by uniprot were classified as likely novel N-glycosylation sites. Volcano plots were generated using the VolcanoShiny app (https://hardingnj.shinyapps.io/volcanoshiny/). PCA and variance analysis were done with the Partek Genomics Suite software. For homologous IgSF-CAM proteins (CNTNs, L1CAMs and NCAMs), HomoloGene and the study from Chen et al. (Chen and Zhou, 2010) were used.

**Granule cell culture**

Cerebellar granule cells were isolated from P7 cerebellum following the Manzini and colleagues protocol (Lee et al., 2009). Cells were kept at 37°C in 24 or 48-well plates for at least 24 hr. To detect expression of proteins at the plasma membrane, the protocol published by Carrodus et al. was followed (Carrodus et al., 2014). The rat monoclonal anti-L1CAM antibody (557.B6; 1:100) has been described (Appel et al., 1995). The anti-NrCAM antibody was purchased from Abcam (AB24344, 1:400). For binding with L1CAM-Fc (10 μg/mL; 777-NC, R and D) and NrCAM-Fc chimeras (10 μg/mL; 2034-NR, R and D), the cells were incubated at 37°C with the chimeras for 4 hr and 10 min, respectively, in granule cell culture media. Both the cell surface protein expression and binding assays were repeated three times with biological replicates. For each experiment, at least 20 neurons per mice were analyzed. The differences measured between control and mutant cells and expressed as a percentage in the main text represent the average of all the repeated experiments. Quantification and normalization were performed using imageJ and TuJ1 staining. For tunicamycin treatment, the cells were incubated for 16 hr with 0.01 μg /mL tunicamycin in culture media prior ICC. For surface coating, 48 or 24-well plates were incubated at 4°C overnight with the coating solution, followed by 3 PBS washes, blocking for 30 min with BSA to inhibit non-specific binding (10 mg/mL) and three more PBS washes. The coatings used were: human recombinant L1CAM (10 μg/mL; 777-NC, R and D), human recombinant NrCAM (5 μg/mL; 2034-NR, R and D), fibronectin (50 μg/mL; F2006, Sigma) and Laminin and poly-D-Lysine (2 μg/mL and 30 μg/mL; 11243217001 and P6407, respectively, Sigma). Cell and neurite dynamics were measured every 3 hr by live cell imaging (Incucyte ZOOM with Neurotracker module, Essen Bioscience). Neurite branching points and neurite length were normalized to the total number of cell body clusters. As long as GCs do not arborize, the neurite branching points parameter provided by the Incucyte software was translated as neurite number. For GCs re-aggregates, cells were isolated and plated for 24 hr in un-coated surfaces (2 × 10^6 cells/cm^2) to promote aggregation. The aggregates were then collected and seeded on the coated surface for 24 hr. Sholl analysis (using the ImageJ plugin) was performed to quantify the
aggregates' neurite number as the number of neurites crossing each circle. For migration analyses, the cells within the same circles used in Sholl analyses but the first circle, were counted (Tanaka et al., 2004). Five different aggregates were quantified per mouse and coating condition.

Human-induced pluripotent stem cells (hiPSCs) SRD5A3−/− generation and culture
iPSCs were derived from hPBMCs from a control male donor via Cyto-Tune Sendai virus reprogramming. Cells were cultured at 37°C on vitronectin-coated (10 μg/mL; 07180, Stem Cell) dishes with mTeSR media (Stem Cell). SRD5A3 KO hiPSCs clones were generated by CRISPR/Cas9. sgRNA (inserted into a GFP-containing PX458 plasmid, Addgene) targeting the first exon of the gene were generated via the CRISPOR website and validated in T293 HEK cells by Sanger sequencing combined with tides analysis (https://tide-calculator.nki.nl/; data not shown). hiPSCs were transfected by nucleofection (Amaza 4D, Lonza) and transfected cells (GFP+) were isolated by FACs (BD FACS Aria II SORP, BD Biosciences) to generate single-cell-of-origin colonies. DNA was extracted from a piece of each colony by ZR-Duet DNA MiniPrep (D7003, Zymo) and sequenced. After selection, SOX2 (1:2000; AB5603, Millipore) and OCT4 (1:100; SC5279, Santa Cruz) immunostaining was used to confirm pluripotency (Figure 5—figure supplement 2). No major chromosomal abnormalities were detected by CGH array (60K, data not shown).

hiPSCs differentiation towards late cortical progenitors (LCPs)
Differentiation of hiPSCs towards LCPs and cortical projection neurons (CPNs) was carried as described by Benchoua and colleagues (Boissart et al., 2013). Briefly, iPSCs colonies were transferred to a non-coated dish with neural induction media (N2B27 with FGF2 and double SMAD inhibition by SB431542 and LDN193189, Stem Cell) for 6 hr and were afterwards transferred onto polyornithine and laminincoated dishes. Following neural rosette formation (12 – 15 days), the cells were passaged onto a gelretex-coated (A1413301, Life Tech) flask (LCPs P1) and were further cultured with N2B27 supplemented with FGF2, EGF and BDNF (Stem Cell). SOX2 and Nestin (1:250; N5413, Sigma) staining confirmed LCPs multipotency. Neuroectodermal origin of the emerging neural progenitor-like cells was assessed by HNK1/P75 FACs staining (data not shown). LCPs were further differentiated towards CPNs by growth factor withdrawal for 15 days. Neuronal identity was assessed with Tuj1 (1:2000; T2200, Sigma) staining (data not shown). WB results were replicated with two different clones of control and SRD5A3 mutant hiPSCs.

Electronic microscopy experiments
P21 En1Cre; Srd5a3−/− (n = 1), Atoh1Cre; Srd5a3−/− (n = 2) and control littermates (n = 3) were perfused with 4% PFA/2% glutaraldehyde. The cerebellum was kept in the same solution for at least one week. Sagittal slices, less than 1 mm thick, were post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer and then dehydrated in ethanol. After 10 min in a 1:2 mixture of epoxy propane and epoxy resin and 10 min in epon, samples were covered by upside down gelatin capsules filled with freshly prepared epoxy resin and polymerized at 60°C for 24 hr. After heat separation, ultrathin sections of 90 nm were cut with an ultra-microtome (Reichert ultracut S), stained with uranyl acetate and Reynold’s lead and observed with a transmission electron microscope (JEOL 1011). Acquisition was performed with a Gatan Orius 1000 CCD camera.

Statistics
For mouse experiments, no statistical methods were used to predetermine sample size. No animals or samples were excluded from the analysis. All mouse experiments included at least two different litters. For mouse behavioral analysis, one way ANOVA was used. Regular enrichment analyses were performed by Fisher exact test (significance 0.05). For multiplicity and NxS motif correlation, the Pearson coefficient was used. For enrichment in highly N-glycosylated proteins, two-tailed Mann-Whitney test was performed. Statistical differences between groups were assessed by two-tailed student t-test. For all statistical tests, confidence intervals were set at 95%. All results are presented in mean value and standard deviation was used to calculate the error bars.
Data availability
Full transcriptomic data is publicly available at ArrayExpress (accession no. E-MTAB-6861). Total proteomics and glycoproteomics data are available via ProteomeXchange with identifier PXD009906.

Acknowledgements
We thank JG Gleeson for the support to generate Srd5a3 conditional mouse and helpful advices. We are grateful to L Goutebroze, F Francis, N Altin, K Siquier, K Radjamani for valuable discussions or technical help. The project is funded by the French National Research Agency ANR-16-CE12-0005-01 (VC), la Fondation pour la Recherche Médicale FRM-DEQ20160334938 (LC), the association Connaître les Syndromes Cérébelleux CSC (VC), the program ERA-NET 643578-EURO-CDG2 and E-Rare-3 (FF and CT), Paris Descartes University BioSPC program (DM-C), Imagine Foundation (DM-C), Imagine International PhD program with Bettencourt-Schueller-foundation (EU). We thank Cochin genomic (GENOMIC), electron microscopy core facilities and the Imagine animal facilities (LEAT) for services and support. We also thank the Animal Histology and Morphology Core Facility of SFR Necker (Inserm US24). We also thank L. Legeai-Mallet and C Colnot for providing the CMV-Cre and the Rosa26-LacZ mouse, respectively and Nicolas Cagnard for statistical advice.

Additional information

Funding

| Funder                                   | Grant reference number          | Author                      |
|------------------------------------------|---------------------------------|-----------------------------|
| Université Paris Descartes               | BioSPC program                  | Daniel Medina-Cano          |
| European Commission                      | ERA-NET 643578-EURO-CDG2        | Christian Thiel             |
|                                         |                                  | François Foulquier          |
| European Commission                      | ERA-NET E-Rare-3                | Christian Thiel             |
|                                         |                                  | François Foulquier          |
| Fondation pour la Recherche Médicale     | FRM-DEQ20160334938              | Laurence Colleaux           |
| Agence Nationale de la Recherche         | ANR-16-CE12-0005-01             | Vincent Cantagrel           |
| Association Connaître les Syndromes Cérébelleux |                          | Vincent Cantagrel           |

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions

Daniel Medina-Cano, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing—original draft; Ekin Ucuncu, Data curation, Formal analysis, Methodology; Lam Son Nguyen, Joanna Lipecka, Christian Thiel, Formal analysis, Methodology; Michael Nicouleau, Data curation, Formal analysis; Jean-Charles Bizot, Data curation, Formal analysis, Supervision; François Foulquier, Data curation, Investigation; Nathalie Lefort, Formal analysis; Catherine Faivre-Sarrailh, Conceptualization, Resources, Supervision, Methodology, Writing—review and editing; Laurence Colleaux, Formal analysis, Funding acquisition, Project administration, Writing—review and editing; Ida Chiara Guerrera, Resources, Data curation, Formal analysis, Supervision, Visualization, Methodology; Vincent Cantagrel, Conceptualization, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Methodology, Writing—original draft, Writing—review and editing

Author ORCIDs

Vincent Cantagrel http://orcid.org/0000-0002-5180-4848

Ethics

Human subjects: Primary human mononuclear cells were isolated from peripheral blood and were obtained using Institutional Review Board-approved consent forms. Induced human pluripotent stem
cells protocols, samples collection and storage were approved by the French research ministry, identification DC 2015-2595 obtained on Sept-05 2016.

Animal experimentation: In this study, animals were used in compliance with the French Animal Care and Use Committee from the Paris Descartes University (APAFIS#961-201506231137361). All behavioural analysis were performed in compliance with the guidelines of the French Ministry of Agriculture for experiments with laboratory animals. The experimental protocol and euthanasia have been approved by the Ethical Committee 27, registered at the French ministry of research.

Decision letter and Author response
Decision letter https://doi.org/10.7554/eLife.38309.026
Author response https://doi.org/10.7554/eLife.38309.027

Additional files

Supplementary files

- Supplementary file 1. Total proteomics. Normalized and log2 transformed intensities of the 1982 proteins detected by total proteomics and their relative abundance levels in the En1-Cre; Srd5a3<sup>−/−</sup> mutant cerebellum.
  
  DOI: https://doi.org/10.7554/eLife.38309.017

- Supplementary file 2. Glycoproteomics. All of the N-glycosylation sites detected in at least 2 out of 4 control samples are included and classified based on their presence in PMID: 20510933 or if they are predicted by uniprot (reference glycoproteomic dataset, spread sheet 1). Sequon analyses on the 1665 N-glycopeptides within the glycoproteomic dataset (spread sheet 2). Gene ontology analyses on the N-glycosylation sites within the reference glycoproteomic dataset predicted by uniprot and/or found in PMID: 20510933 and on the N-glycosylation sites not present in none of them (novel sites, spread sheet 3). 1404 N-glycopeptides detected in at least 3 out of 4 control samples and their corresponding abundance levels in the mutant samples; the intensity for each N-glycopeptide are log2 transformed (comparative glycoproteomics, spread sheet 4). Sequon analyses on the comparative glycoproteomics dataset (spread sheet 5).
  
  DOI: https://doi.org/10.7554/eLife.38309.018

- Supplementary file 3. Total proteomics and glycoproteomics. List, normalized intensities and relative abundance of the 24 peptides detected in total proteomics and with decreased abundance in glycoproteomics in the mutant.
  
  DOI: https://doi.org/10.7554/eLife.38309.019

- Transparent reporting form
  
  DOI: https://doi.org/10.7554/eLife.38309.020

Data availability

Expression microarray data are publicly available at ArrayExpress accession number E-MTAB-6861. Proteomics and Glycoproteomics data are included within supplementary files 1, 2 and 3 and full proteomics and glycoproteomics data are available via ProteomeXchange with identifier PXD009906.

The following datasets were generated:

| Author(s)               | Year | Dataset title                                                                 | Dataset URL                                                                 | Database, license, and accessibility information |
|-------------------------|------|-------------------------------------------------------------------------------|-----------------------------------------------------------------------------|-----------------------------------------------|
| Medina-Cano D, Lipecka J, Guerrera IC, Cantagrel V | 2018 | GlycoProteomics in CDG (congenital disorder of glycosylation)                  | https://www.ebi.ac.uk/pride/archive/projects/PXD009906 | Publicly available at EBI PRIDE (accession no. PXD009906) |
| Medina-Cano D., Cantagrel V | 2018 | Transcriptomic analysis of the developing cerebellum in a mouse model for SRD5A3-CDG | https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6861/ | Publicly available at EMBL-EBI Array Express (accession no. E-MTAB-6861) |
References

Agrawal P, Fontanals-Cirera B, Sokolova E, Jacob S, Vaiana CA, Argibay D, Davalos V, McDermott M, Nayak S, Darvishian F, Castillo M, Uebereinde B, Osman I, Fenyo D, Mahal LK, Hernando E. 2017. A systems biology approach identifies FUT8 as a driver of melanoma metastasis. Cancer Cell 31:804–819. DOI: https://doi.org/10.1016/j.ccell.2017.05.007, PMID: 28606658

Akizu N, Cantagrel V, Schroth J, Cai N, Vaux K, McCloskey D, Naviaux RK, Van Vleet J, Fenstermaker AG, Silhavy JL, Scheliga JS, Toyama K, Morisaki H, Sonmez FM, Celep F, Orabey A, Zaki MS, Al-Baradie R, Faqeih EA, Saleh MA, et al. 2013. AMPD2 regulates GTP synthase and is mutated in a potentially treatable neurodegenerative brainstem disorder. Cell 154:505–517. DOI: https://doi.org/10.1016/j.cell.2013.07.005, PMID: 23911318

Appel F, Holm J, Consience JF, von Bohlen und Halbach F, Faissner A, James P, Schachner M. 1995. Identification of the border between fibronectin type III homologous repeats 2 and 3 of the neural cell adhesion molecule L1 as a neurite outgrowth promoting and signal transducing domain. Journal of Neurobiology 28:297–312. DOI: https://doi.org/10.1002/jneu.480280304, PMID: 8568512

Berglund EO, Murali KK, Fredette B, Sekerková G, Marturano B, Weber L, Mugnaini E, Ranscht B. 1999. Ataxia and abnormal cerebellar microorganization in mice with ablated contactin gene expression. Neuron 24:739–750. DOI: https://doi.org/10.1016/S0896-6273(00)81126-5, PMID: 10595523

Bizzoca A, Virginitino D, Lorusso L, Buttiglione M, Yoshida L, Polizzi A, Tattoli M, Cagiano R, Rossi F, Kozlov S, Furley A, Gennarini G. 2003. Transgenic mice expressing F3/contactin from the TAG-1 promoter exhibit developmentally regulated changes in the differentiation of cerebellar neurons. Development 130:29–43. DOI: https://doi.org/10.1242/dev.001813, PMID: 12441289

Blank C, Smith LA, Hammer DA, Fehrenbach M, Delisser HM, Perez E, Sullivan KE. 2006. Recurrent infections and immunological dysfunction in congenital disorder of glycosylation Ia (CDG Ia). Journal of Inherited Metabolic Disease 29:592. DOI: https://doi.org/10.1054/jimd.2005.006-0275-2, PMID: 16826448

Boissart C, Poulet A, Georges P, Darville H, Julita E, Delorme R, Bourgeron T, Peschanski M, Benchoua A. 2013. Differentiation from human pluripotent stem cells of cortical neurons of the superficial layers amenable to psychiatric disease modeling and high-throughput drug screening. Translational Psychiatry 3:e294. DOI: https://doi.org/10.1038/tp.2013.71, PMID: 23962924

Burda P, Aebi M. 1999. The dolichol pathway of N-linked glycosylation. Biochimica et Biophysica Acta (BBA) - General Subjects 1426:239–257. DOI: https://doi.org/10.1016/S0304-4165(98)00127-5, PMID: 9878760

Cantagrel V, Lefeber DJ, Ng BG, Guan Z, Silhavy JL, Bielas SL, Lehe L, Hombauer H, Adamowicz M, Swiezewska E, De Brouwer AP, Blümel P, Sykut-Cegielska J, Houliston S, Swistun D, Ali BR, Dobyns WB, Babovic-Vuksanovic D, van Bokhoven H, Wevers RA, et al. 2010. SRD5A3 is required for converting polyisoprenol to dolichol and is mutated in a congenital glycosylation disorder. Cell 142:203–217. DOI: https://doi.org/10.1016/j.cell.2010.06.001, PMID: 20637498

Cantagrel V, Lefeber DJ. 2011. From glycosylation disorders to dolichol biosynthesis defects: a new class of metabolic diseases. Journal of Inherited Metabolic Disease 34:859–867. DOI: https://doi.org/10.1007/s10545-011-9301-0, PMID: 21384228

Cao J, Guo S, Ariai K, Lo EH, Ning M. 2013. Studying extracellular signaling utilizing a glycoproteomic approach: lectin blot surveys, a first and important step. Methods in Molecular Biology 1010:227–233. DOI: https://doi.org/10.1007/978-1-62703-426-5_15, PMID: 23625563

Carrodot NL, Teng KS-L, Munro KM, Kennedy MJ, Gunnerensen JM. 2014. Differential labeling of Cell-surface and internalized proteins after antibody feeding of live cultured neurons. Journal of Visualized Experiments 84: e51193. DOI: https://doi.org/10.3791/51193

Chan B, Clasquin M, Smolen GA, Histen G, Powe J, Chen Y, Lin Z, Lu C, Liu Y, Cang Y, Yan Z, Xiao Y, Thompson TL, Singleton C, Dorsch M, Silverman L, Su SM, Freeze HH, Jin S. 2016. A mouse model of a human congenital disorder of glycosylation caused by loss of PMM2. Human Molecular Genetics 25:2182–2193. DOI: https://doi.org/10.1093/hmg/ddw085, PMID: 27053713

Chavan M, Lennarz W. 2006. The molecular basis of coupling of translocation and N-glycosylation. Trends in Biochemical Sciences 31:17–20. DOI: https://doi.org/10.1016/j.tibs.2005.11.010, PMID: 16356726

Chen L, Zhou S. 2010. "CRASH"ing with the worm: insights into LIN12/LIN22 functions and mechanisms. Developmental Dynamics: An Official Publication of the American Association of Anatomists 239:1490–1501. DOI: https://doi.org/10.1002/dvdy.22269, PMID: 20225235

Cherepanova NA, Gilmore R. 2016. Mammalian cells lacking either the cotranslational or posttranslational oligosaccharyltransferase complex display substrate-dependent defects in asparagine linked glycosylation. Scientific Reports 6:20946. DOI: https://doi.org/10.1038/srep20946, PMID: 26864433

Cline A, Gao N, Flanagan-Stee H, Sharma V, Rosa S, Sonon R, Azadi P, Sadler KC, Freeze HH, Lehman MA, Steet R. 2012. A zebrafish model of PMM2-CDG reveals altered neurogenesis and a substrate-accumulation mechanism for N-linked glycosylation deficiency. Molecular Biology of the Cell 23:4175–4187. DOI: https://doi.org/10.1091/mbc.e12-05-0411, PMID: 22956764

Çolakoğlu G, Bergstrom-Tyrberg U, Berglund EO, Ranscht B. 2014. Contactin-1 regulates myelination and nodal/paranodal domain organization in the central nervous system. PNAS 111:E394–E403. DOI: https://doi.org/10.1073/pnas.1313769110, PMID: 24385581

Dahmane N, Ruiz i Altaba A. 1999. Sonic hedgehog regulates the growth and patterning of the cerebellum. Development 126:3089–3100. PMID: 10375501
Demyanenko GP, Tsai AY, Maness PF. 1999. Abnormalities in neuronal process extension, hippocampal development, and the ventricular system of L1 knockout mice. The Journal of Neuroscience 19:4907–4920. DOI: https://doi.org/10.1523/JNEUROSCI.19-12-04907.1999, PMID: 10366625

Demyanenko GP, Riday TT, Tran TS, Dalal J, Damell EP, Brennanman LH, Sakurai T, Grumet M, Philpot BD, Maness PF. 2011. NrCAM deletion causes topographic misrouting of thalamocortical axons to the visual cortex and disrupts visual acuity. Journal of Neuroscience 31:1545–1558. DOI: https://doi.org/10.1523/JNEUROSCI.4467-10.2011, PMID: 21273439

Dennis JW, Nabi IR, Demetriou M. 2009. Metabolism, cell surface organization, and disease. Cell 139:1229–1241. DOI: https://doi.org/10.1016/j.cell.2009.12.008, PMID: 20064370

Dequidt C, Daniolet L, Alberts P, Galli T, Choquet D, Thoumine O. 2007. Fast turnover of L1 adhesions in neuronal growth cones involving both surface diffusion and exo/endocytosis of L1 molecules. Molecular Biology of the Cell 18:3131–3143. DOI: https://doi.org/10.1091/mbc.e06-12-1101, PMID: 17538021

Falk J, Bonnon C, Girault JA, Faivre-Sarrailh C. 2002. F3/contactin, a neuronal cell adhesion molecule implicated in axogenesis and myelination. Biology of the Cell 94:327–334. DOI: https://doi.org/10.1016/S0248-4900(02)00006-0, PMID: 12509940

Frazn E, D’Hooge R, Van Camp G, Verhoeve M, Sijbers J, Reyniers E, Soriano P, Kamiguchi H, Willemsen R, Koekkoek SK, De Zeeuw CI, De Deyn PP, Van der Linden A, Lemmon V, Kooy RF, Willems PJ. 1998. L1 knockout mice show dilated ventricles, vermis hypoplasia and impaired exploration patterns. Human Molecular Genetics 7:999–1009. DOI: https://doi.org/10.1093/hmg/7.6.999, PMID: 9580664

Freeze HH, Chong JX, Bamshad MJ, Ng BG. 2014. Solving glycosylation disorders: fundamental approaches reveal complicated pathways. The American Journal of Human Genetics 94:161–175. DOI: https://doi.org/10.1016/j.ajhg.2013.10.024, PMID: 24507773

Freeze HH, Eklund EA, Ng BG, Patterson MC. 2015a. Neurological aspects of human glycosylation disorders. Annual Review of Neuroscience 38:105–125. DOI: https://doi.org/10.1146/annurev-neuro-071714-034019, PMID: 25840006

Freeze HH, Schachter H, Kinoshita T. 2015b. Genetic Disorders of Glycosylation. In: rd A, Varki R. D, Cummings J. D, Esko P, Stanley G. W, Hart M, Aebi A. G, Darvill T, Kinoshita N. H, Packer J. H, Prestegard R. L. S. chnaar, J. D, Eklund EA, Ng BG, Patterson MC. 2015a. Neurological aspects of human glycosylation disorders. Annual Review of Neuroscience 38:105–125. DOI: https://doi.org/10.1146/annurev-neuro-071714-034019, PMID: 25840006

Gavel Y, Heijne G von. 1990. Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. “Protein Engineering, Design and Selection” 3:433-442. DOI: https://doi.org/10.1016/0267-889X(90)90009-G

Gründahl JE, Guan Z, Rust S, Reunert J, Müller B, Du Chesne, Zerres K, Rudnik-Schöneborn S, Ortiz-Brüchle N, Häusler MG, Siedlecka J, Swiezewska E, Raetz CR, Marquardt T. 2012. Life with too much polyprenol: polyprenol reductase deficiency. Molecular Genetics and Metabolism 105:642–651. DOI: https://doi.org/10.1002/mgen.201112.017, PMID: 22304929

Haueptle MA, Welti M, Troxler H, Hülsmeier AJ, Imbach T, Hennet T. 2011. Improvement of dolichol-linked oligosaccharide biosynthesis by the squalene synthase inhibitor zaragozic acid. Journal of Biological Chemistry 286:6085–6091. DOI: https://doi.org/10.1074/jbc.M110.165795, PMID: 21183681

He P, Ng BG, Losfeld ME, Zhu W, Freeze HH. 2012. Identification of intercellular cell adhesion molecule 1 (ICAM-1) as a hypoglycosylation marker in congenital disorders of glycosylation cells. Journal of Biological Chemistry 287:18210–18217. DOI: https://doi.org/10.1074/jbc.M112.355677, PMID: 22496445

He P, Srirkrihna G, Freeze HH. 2014. N-glycosylation deficiency reduces ICAM-1 induction and impairs inflammatory response. Glyobiology 24:392–398. DOI: https://doi.org/10.1093/glycob/cwu006, PMID: 24474243

Hörstkotte R, Schachner M, Magyar JP, Vorherr T, Schmitz B. 1993. The fourth immunoglobulin-like domain of NCAM contains a carbohydrate recognition domain for oligomannosidic glycans implicated in association with L1 and neurite outgrowth. The Journal of Cell Biology 121:1409–1421. DOI: https://doi.org/10.1083/jcb.121.6.1409, PMID: 8509458

Hortsch M. 2000. Structural and functional evolution of the L1 family: are four adhesion molecules better than one? Molecular and Cellular Neuroscience 15:1–10. DOI: https://doi.org/10.1006/mcne.1999.0809, PMID: 10662501

Hülsmeier AJ, Paesold-Burda P, Hennet T. 2007. N-glycosylation site occupancy in serum glycoproteins influenced by multiple reaction monitoring liquid chromatography-mass spectrometry. Molecular & Cellular Proteomics 6:2132–2138. DOI: https://doi.org/10.1074/mcp.M700361-MCP200, PMID: 17823199

Jaeken J, Péanne R. 2017. What is new in CDG? Journal of Inherited Metabolic Disease 40:569–586. DOI: https://doi.org/10.1038/s41436-017-0050-0, PMID: 28484880

Kara B, Ayhan Ö, Gökçay G, Başboğaoğlu N, Tolun A. 2014. Adult phenotype and further phenotypic variability in SRDSA3-CDG. BMC Medical Genetics 15:10. DOI: https://doi.org/10.1186/1471-2350-15-10, PMID: 24433453

Katji J, Loers G, Kleene R, Karl N, Schmidt C, Buck F, Zmijewski JW, Jakovec KI, Preisssner KT, Schachter M. 2014. Interaction of the cell adhesion molecule CHL1 with vitronectin, integrins, and the plasminogen activator inhibitor-2 promotes CHL1-induced neurite outgrowth and neuronal migration. Journal of Neuroscience 34:14606–14623. DOI: https://doi.org/10.1523/JNEUROSCI.3280-13.2014, PMID: 25355214
Kerjan G, Dolan J, Haumairte C, Schneider-Maunoury S, Fujisawa H, Mitchell KJ, Chédotal A. 2005. The transmembrane semaphorin Sema6A controls cerebellar granule cell migration. Nature Neuroscience 8:1516–1524. DOI: https://doi.org/10.1038/nn1555, PMID: 16205717

Kiefel H, Bondong S, Hazin J, Ridinger J, Schirmer U, Riedle S, Altevogt P. 2012. L1CAM: a Major driver for tumor cell invasion and motility. Cell Adhesion & Migration 6:374–384. DOI: https://doi.org/10.4161/cam.20832, PMID: 22796939

Kazio臻 LF, Budding D, Andreason N, D’Arrigo S, Bulgheroni S, Imamizu H, Ito M, Manto M, Marvel C, Parker K, Pezzulo G, Ramnani N, Riva D, Schmahmann J, Vandervert L, Yamazaki T. 2014. Consensus paper: the cerebellum’s role in movement and cognition. The Cerebellum 13:151–177. DOI: https://doi.org/10.1007/s12311-013-0511-x, PMID: 23996631

Kretzer NM, Thiesen DJ, Tussiwand R, Briseño CG, Grajales-Reyes GE, Wu X, Durai V, Albring J, Bagadia P, Murphy TL, Murphy KM. 2016. RAB43 facilitates cross-presentation of cell-associated antigens by CD8+ dendritic cells. The Journal of Experimental Medicine 213:2871–2883. DOI: https://doi.org/10.1084/jem.20160597, PMID: 27899433

Lau KS, Partridge EA, Grigorian A, Silvescu CI, Reinhold VN, Demetriou M, Dennis JW. 2007. Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. Cell 129:123–134. DOI: https://doi.org/10.1016/j.cell.2007.01.049, PMID: 17418791

Lee HY, Greene LA, Mason CA, Manzini MC. 2009. Isolation and culture of Post-Natal mouse cerebellar granule neuron progenitor cells and neurons. Journal of Visualized Experiments 23. DOI: https://doi.org/10.3791/990

Lieberoth A, Splitztoeßler F, Kataghillimath N, Jakovecvi, I, Loers G, Ranscht B, Karagoezos D, Schachner M, Kleene R. 2009. Lewis(x) and alpha2,3-sialyl glycans and their receptors TAG-1, Contactin, and L1 mediate CD24-dependent neurite outgrowth. Journal of Neuroscience 29:6677–6690. DOI: https://doi.org/10.1523/JNEUROSCI.4361-08.2009, PMID: 19458237

Lipecka J, Chhuon C, Bourderiou M, Bessard MA, van Endert P, Edelman A, Guerrera IC. 2016. Sensitivity of mass spectrometry analysis depends on the shape of the filter unit used for filter aided sample preparation (FASP). Proteomecs 16:1852–1857. DOI: https://doi.org/10.1002/pmc.201601003, PMID: 27219663

Lutz D, Loers G, Kleene R, Ozeen I, Kataria H, Kataghillimath N, Braren I, Harauz G, Schachner M. 2014. Myelin basic protein cleaves cell adhesion molecule L1 and promotes neuritogenesis and cell survival. Journal of Biological Chemistry 289:13503–13518. DOI: https://doi.org/10.1074/jbc.M113.530238, PMID: 24671420

Maness PF, Schachner M. 2007. Neural recognition molecules of the immunoglobulin superfamily: signaling transducers of axon guidance and neuronal migration. Nature Neuroscience 10:19–26. DOI: https://doi.org/10.1038/nn1827, PMID: 17189949

Matei V, Pauley S, Kaing S, Rowitch D, Beisel KW, Morris K, Feng F, Jones K, Lee J, Fritzsch B. 2005. Smaller inner ear sensory epithelia in Neurog 1 null mice are related to earlier hair cell cycle exit. Developmental Dynamics 234:633–650. DOI: https://doi.org/10.1002/dvdy.20551, PMID: 16145671

Metzger D, Clifford J, Chiba H, Champon P. 1995. Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. PNAS 92:6991–6995. DOI: https://doi.org/10.1073/pnas.92.15.6991, PMID: 7624356

Morava E, Wosik HN, Syktu-Cegielska J, Adamowicz M, Guillard M, Wevers RA, Lefeber DJ, Cruesberg JR. 2009. Ophthalmomological abnormalities in children with congenital disorders of glycosylation type I. British Journal of Ophthalmology 93:350–354. DOI: https://doi.org/10.1136/bjo.2008.145359, PMID: 19019927

Morava E, Wevers RA, Cantagrel V, Hoeftsoot LH, Al-Gazali L, Schoots J, van Rooij A, Huijbren K, van Ravenswaaij-Arts CM, Jongmans MC, Syktu-Cegielska J, Hoffmann GF, Bluemel P, Adamowicz M, van Reeuwijk J, Ng BG, Bergman JE, van Bokhoven H, Korner C, Babovic-Vukanovic D, et al. 2010. A novel cerebello-ocular syndrome with abnormal glycosylation due to abnormalities in dolichol metabolism. J Neuroophthalmology 30:350–354. DOI: https://doi.org/10.1016/j.jnt.2010.03.002, PMID: 20960283

Ng BG, Freeze HH. 2018. Perspectives on glycosylation and its congenital disorders. Trends in Genetics : TIG 34:466–476. DOI: https://doi.org/10.1016/j.tig.2018.03.002, PMID: 29606283

Nguyen H, Ostendorf AP, Satz JS, Westra S, Ross-Barta SE, Campbell KP, Moore SA. 2013. Giall scaffold required for cerebellar granule cell migration is dependent on dystroglycan function as a receptor for basement membrane proteins. Acta Neuropathologica Communications 1:58. DOI: https://doi.org/10.1186/2051-5960-1-58, PMID: 24252195

Park EJ, Grabiška KA, Guan Z, Stránecky V, Hartmannová H, Hodařová K, Barešová V, Sovová J, Jozsef L, Ondrušková N, Hanšiková H, Honzik T, Žeman J, Hůlková H, Wen R, Knioh S, Sessa WC. 2014. Mutation of Nogo-B receptor, a subunit of cis-prenyltransferase, causes a congenital disorder of glycosylation. Cell Metabolism 20:448–457. DOI: https://doi.org/10.1016/j.cmet.2014.06.016, PMID: 25066056

Pereira PL, Magnol L, Sahün I, Broult V, Duchon A, Prandini P, Gruart A, Bizot JC, Chadevaux-Vekemans B, Deutsch S, Trovero F, Delgado-Garcia J M, Antonarakis SE, Dierssen M, Herault Y. 2009. A new mouse model for the trisomy of the Abcg1-U2af1 region reveals the complexity of the combinatorial genetic code of down syndrome. Human Molecular Genetics 18:4756–4769. DOI: https://doi.org/10.1093/hmg/ddp438, PMID: 19783846
Pogoriler J, Millen K, Utset M, Du W. 2006. Loss of cyclin D1 impairs cerebellar development and suppresses medulloblastoma formation. Development 133:3929–3937. DOI: https://doi.org/10.1242/dev.02556, PMID: 16943274

Poljak K, Selevsek N, Ngwa E, Grossmann J, Losfeld ME, Aebi M. 2018. Quantitative profiling of N-linked glycosylation machineries in yeast Saccharomyces cerevisiae. Molecular & Cellular Proteomics 17:18–30. DOI: https://doi.org/10.1074/mcp.RA117.000996, PMID: 28993419

Pollerberg GE, Thelen K, Theiss MO, Hochlehnert BC. 2013. The role of cell adhesion molecules for navigating axons: density matters. Mechanisms of Development 130:359–372. DOI: https://doi.org/10.1016/j.mod.2012.11.002, PMID: 23183391

Richard E, Vega AI, Pérez B, Roche C, Velázquez R, Ugarte M, Pérez-Cerdà C. 2009. Congenital disorder of glycosylation Ia: new differentially expressed proteins identified by 2-DE. Biochemical and Biophysical Research Communications 379:267–271. DOI: https://doi.org/10.1016/j.bbrc.2008.12.036, PMID: 19101518

Ron D, Walter P. 2007. Signal integration in the endoplasmic reticulum unfolded protein response. Nature Reviews Molecular Cell Biology 8:519–529. DOI: https://doi.org/10.1038/nrn2199, PMID: 17565364

Rujano MA, Cannata Serio M, Panasyuk G, Péanne R, Reunert J, Rymen D, Hauser V, Park JH, Freisinger P, Souche E, Guida MC, Maier EM, Wada Y, Jäger S, Krogan NJ, Kretz O, Nobire S, García P, Quelhas D, Bird TD, et al. 2017. Mutations in the X-linked ATP6AP2 cause a glycosylation disorder with autophagic defects. The Journal of Experimental Medicine 214:3707–3729. DOI: https://doi.org/10.1084/jem.20170453, PMID: 29127204

Sakurai T, Lustig M, Babiarz J, Furley AJ, Tait S, Brophy PJ, Brown SA, Brown LY, Mason CA, Grumet M. 2001. Overlapping functions of the cell adhesion molecules Nr-CAM and L1 in cerebellar granule cell development. The Journal of Cell Biology 154:1259–1274. DOI: https://doi.org/10.1083/jcb.200014122, PMID: 11564762

Schiff M, Roda C, Monin ML, Arion A, Barth M, Bednarek N, Bidet M, Bloch C, Boddart N, Borgel D, Brasser A, Brice A, Brunelle A, Buissinière R, Chabrol B, Chevalier MC, Cormier-Daire V, De Barace C, De Maistre E, De Saint-Martin A, et al. 2017. Clinical, laboratory and molecular findings and long-term follow-up data in 96 French patients with PPM2-C-DCG (phosphomannomutase 2-congenital disorder of glycosylation) and review of the literature. Journal of Medical Genetics 54:843–851. DOI: https://doi.org/10.1136/jmedgenet-2017-104903, PMID: 28954837

Schwarz V, Pan J, Volmier-Irsch S, Hutter H. 2009. IgCAMs redundantly control axon navigation in Caenorhabditis elegans. Neural Development 4:13. DOI: https://doi.org/10.1186/1749-8104-4-13, PMID: 19341471

Scott H, Panin VM. 2014a. N-glycosylation in regulation of the nervous system. Advances in Neurobiology 9:367–394. DOI: https://doi.org/10.1007/978-1-4939-1154-7_17, PMID: 25151388

Scott H, Panin VM. 2014b. The role of protein N-glycosylation in neural transmission. Glycobiology 24:407–417. DOI: https://doi.org/10.1093/glycob/cwu015, PMID: 24643084

Sgaier SK, Lao Z, Villanueva MP, Berensteyn F, Stephen D, Turnbull RK, Joyner AL. 2007. Genetic subdivision of the tectum and cerebellum into functionally related regions based on differential sensitivity to engrafted proteins. Development 134:2325–2335. DOI: https://doi.org/10.1242/dev.000620, PMID: 17537797

Sonderegger P. 1997. Axonin-1 and NgCAM as "recognition" components of the pathway sensor apparatus of growth cones: a synopsis. Cell and Tissue Research 290:429–439. DOI: https://doi.org/10.1007/s004410050950, PMID: 9321707

Stoeckli ET, Sonderegger P, Pollerberg GE, Landmesser LT. 1997. Interference with axonin-1 and NrCAM interactions unmasks a floor-plate activity inhibitory for commissural axons. Neuron 18:209–221. DOI: https://doi.org/10.1016/S0896-6273(00)80262-7, PMID: 9052792

Stoeckli ET. 2010. Neural circuit formation in the cerebellum is controlled by cell adhesion molecules of the Contactin family. Cell Adhesion & Migration 4:523–526. DOI: https://doi.org/10.4161/cam.4.4.12733, PMID: 20622526

Sytnyk V, Leshchyns’ka I, Schachner M. 2017. Neural cell adhesion molecules of the immunoglobulin superfamily regulate synapse formation, maintenance, and function. Trends in Neurosciences 40:295–308. DOI: https://doi.org/10.1016/j.tins.2017.03.003, PMID: 28359630

Tanaka T, Serneo FF, Higgins C, Gambello MJ, Wynshaw-Boris A, Gleeson JG. 2004. Lis1 and doublecortin function with dynein to mediate coupling of the nucleus to the centrosome in neuronal migration. The Journal of Cell Biology 165:709–721. DOI: https://doi.org/10.1083/jcb.200309025, PMID: 15173193

Tuyusuz B, Pehlivan D, Özkoç A, Jhangiani S, Muzny DM, Lupski JR, Gibbs R, Jaeken J. 2016. Phenotypic expansion of congenital disorder of glycosylation due to SRD5A3 null mutation. JIMD Reports 26:7–12. DOI: https://doi.org/10.1007/1078904_2015_478, PMID: 26219881

Vogel C, Chothia C. 2006. Protein family expansions and biological complexity. PLoS Computational Biology 2:e48. DOI: https://doi.org/10.1371/journal.pcbi.0020048, PMID: 16735546

Wei CH, Ryu SE. 2012. Homophilic interaction of the L1 family of cell adhesion molecules. Experimental & Molecular Medicine 44:413–423. DOI: https://doi.org/10.3858/emm.2012.44.7.050, PMID: 22573111

Weinhold B, Seidenfaden R, Röckle I, Mühlenhoff M, Schertzinger F, Conzelmann S, Marth JD, Gerardy-Schahn R, Hildebrandt H. 2005. Genetic ablation of polysialic acid causes severe neurodevelopmental defects rescued by deletion of the neural cell adhesion molecule. Journal of Biological Chemistry 280:42971–42977. DOI: https://doi.org/10.1074/jbc.M511097200, PMID: 16267048

Welti M. 2013. Regulation of dolichol-linked glycosylation. Glycoconjugate Journal 30:51–56. DOI: https://doi.org/10.1007/s10719-012-9417-y, PMID: 22717794
Wheeler PG, Ng BG, Sanford L, Sutton VR, Bartholomew DW, Pastore MT, Bamshad MJ, Kircher M, Buckingham KJ, Nickerson DA, Shendure J, Freeze HH. 2016. SRD5A3-CDG: Expanding the phenotype of a congenital disorder of glycosylation with emphasis on adult onset features. American Journal of Medical Genetics Part A 170:3165–3171. DOI: https://doi.org/10.1002/ajmg.a.37875, PMID: 27480077

Węsniewski JR, Zougman A, Nagaraj N, Mann M. 2009. Universal sample preparation method for proteome analysis. Nature Methods 6:359–362. DOI: https://doi.org/10.1038/nmeth.1322, PMID: 19377485

Xenaki D, Martin IB, Yoshida L, Ohyama K, Gennarini G, Grumet M, Sakurai T, Furley AJ. 2011. F3/contactin and TAG1 play antagonistic roles in the regulation of sonic hedgehog-induced cerebellar granule neuron progenitor proliferation. Development 138:519–529. DOI: https://doi.org/10.1242/dev.051912, PMID: 21205796

Xu J. 2005. Preparation, culture, and immortalization of mouse embryonic fibroblasts. Current Protocols in Molecular Biology 28:21.

Zhao L, Rosales C, Seburn K, Ron D, Ackerman SL. 2010. Alteration of the unfolded protein response modifies neurodegeneration in a mouse model of Marinesco-Sjögren syndrome. Human Molecular Genetics 19:25–35. DOI: https://doi.org/10.1093/hmg/ddp464, PMID: 19801575

Zielinska DF, Gnad F, Węsniewski JR, Mann M. 2010. Precision mapping of an in vivo N-glycoproteome reveals rigid topological and sequence constraints. Cell 141:897–907. DOI: https://doi.org/10.1016/j.cell.2010.04.012, PMID: 20510933

Zielinska DF, Gnad F, Schropp K, Węsniewski JR, Mann M. 2012. Mapping N-glycosylation sites across seven evolutionarily distant species reveals a divergent substrate proteome despite a common core machinery. Molecular Cell 46:542–548. DOI: https://doi.org/10.1016/j.molcel.2012.04.031, PMID: 22633491