The leukodystrophy protein FAM126A (hyccin) regulates PtdIns(4)P synthesis at the plasma membrane

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Genetic defects in myelin formation and maintenance cause leukodystrophies, a group of white matter diseases whose mechanistic underpinnings are poorly understood\textsuperscript{1,2}. Hypomyelination and congenital cataract (HCC), one of these disorders, is caused by mutations in FAM126A, a gene of unknown function\textsuperscript{3}. We show that FAM126A, also known as hyccin, regulates the synthesis of phosphatidylinositol 4-phosphate (PtdIns(4)P), a determinant of plasma membrane identity\textsuperscript{4–6}. HCC patient fibroblasts exhibit reduced PtdIns(4)P levels. FAM126A is an intrinsic component of the plasma membrane phosphatidylinositol 4-kinase complex that comprises PI4KIII\textalpha{} and its adaptors TTC7 and EFR3 (refs 5,7). A FAM126A–TTC7 co-crystal structure reveals an all-\alpha{}-helical heterodimer with a large protein–protein interface and a conserved surface that may mediate binding to PI4KIII\textalpha{}.

Absence of FAM126A, the predominant FAM126 isoform in oligodendrocytes, destabilizes the PI4KIII\textalpha{} complex in mouse brain and patient fibroblasts. We propose that HCC pathogenesis involves defects in PtdIns(4)P production in oligodendrocytes, whose specialized function requires massive plasma membrane expansion and thus generation of PtdIns(4)P and downstream phosphoinositides\textsuperscript{8–11}. Our results point to a role for FAM126A in supporting myelination, an important process in development and also following acute exacerbations in multiple sclerosis\textsuperscript{12–14}.

Phosphoinositides are low-abundance anionic membrane phospholipids that play critical roles in many physiological processes\textsuperscript{15,16}. At the plasma membrane, a major phosphoinositide is PtdIns(4)P, which has direct signalling roles and in addition serves as the precursor of two other plasma membrane-enriched phosphoinositides with major signalling functions in this membrane, PtdIns(4,5)\text{P}_2 and PtdIns(3,4,5)\text{P}_3 (refs 4,17). In oligodendrocytes and Schwann cells, these lipids regulate several steps in the biogenesis and maintenance of myelin, including the recruitment of myelin basic protein (MBP) to the plasma membrane by PtdIns(4,5)\text{P}_2 and the promotion of myelin growth by PtdIns(3,4,5)\text{P}_3 (refs 8–11).

Phosphorylation of phosphatidylinositol (PtdIns) to generate PtdIns(4)P in the plasma membrane is mediated by PtdIns-4-kinase Type III\textalpha{} (PI4KIII\textalpha{}) Stt4 in yeast\textsuperscript{18,19}. The properties, targeting mechanisms and regulation of this enzyme have only recently come into focus. Two factors required for its localization at the plasma membrane have been described\textsuperscript{17} and structurally characterized\textsuperscript{20}: EFR3 and TTC7 (Efr3 and Ypp1 in yeast).

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To identify additional regulators of PI4KIII\(\alpha\), we used quantitative interaction proteomics. We immunoprecipitated either stably expressed TTC7B–GFP or GFP, followed by protease digestion and mass spectrometry analysis, to identify candidate TTC7B-binding proteins (Fig. 1a and Supplementary Table 1). These experiments identified the known TTC7B interaction partners PI4KIII\(\alpha\), EFR3A and EFR3B. Among the other hits, two of the most significantly enriched candidates were the paralogues FAM126A, also known as hyccin, and FAM126B, both of which were also identified in similar experiments using EFR3A–GFP or EFR3B–GFP as the bait (Supplementary Fig. 1a).

Mutations in FAM126A that lead to loss of the FAM126A protein cause a recessive leukoencephalopathy termed hypomyelination and congenital cataract\(^3\) (HCC). Manifestations of this condition, which include progressive neurological impairment, mild to moderate cognitive defects, and peripheral neuropathy, stem from progressive neurological impairment, mild to moderate cognitive defects, and peripheral neuropathy, stem from progressive neurological impairment, mild to moderate cognitive defects, and peripheral neuropathy, stem from progressive neurological impairment, mild to moderate cognitive defects, and peripheral neuropathy, stem from progressive neurological impairment, mild to moderate cognitive defects, and peripheral neuropathy, stem from progressive neurological impairment, mild to moderate cognitive defects, and peripheral neuropathy, stem from progressive neurological impairment, mild to moderate cognitive defects, and peripheral neuropathy, stem from progressive neurological impairment, mild to moderate cognitive defects, and peripheral neuropathy, stem from progressive neurological impairment, mild to moderate cognitive defects, and peripheral neuropathy. Indeed, all of these proteins were the strongest hits in analogous quantitative proteomics experiments (Fig. 1a and Supplementary Table 1) and were highly enriched in immunoblot analysis of FAM126A–GFP (bottom), from a label-free proteomics analysis of anti-GFP immunoprecipitates of HEK 293T cells stably expressing TTC7B–GFP, FAM126A–GFP or GFP alone. The logarithmic ratios of protein intensities are plotted against negative logarithmic P-values of two-tailed Student’s t-test, equal variance, performed from three independent experiments. The red dashed line (significance, 0.05) separates specifically interacting proteins (top right portion of plot) from background. Selected top hits are indicated with black dots (bait is indicated in green), and all specific interactors are reported in Supplementary Table 1.
poorly conserved carboxy-terminal tail predicted to be disordered (FAM126A-C, residues 290–521; Fig. 1c). Co-immunoprecipitation experiments of GFP-tagged full-length FAM126A, FAM126A-N or FAM126A-C with differentially tagged PI4KIIIα, TTC7B and EFR3B revealed an interaction of all of these components with full-length FAM126A and FAM126A-N (Fig. 1d). Note that the apparent more robust interaction of these proteins with FAM126A-N than with full-length FAM126A (Fig. 1d, lanes 6 and 7) reflects higher levels of FAM126A-N in the total lysate. Interestingly, overexpression of FAM126A-N led to a marked increase in levels of transfected TTC7B in total lysate (Fig. 1d, lane 3), suggesting a stabilizing interaction between these two proteins, as further confirmed by experiments described below.

We next examined whether, as would be expected for a direct TTC7 interactor, FAM126A-N localizes to the plasma membrane when co-expressed with other PI4KIIIα complex subunits. Using confocal microscopy, we found that GFP-tagged FAM126A-N and full-length FAM126A were localized to the cytosol in HeLa and COS-7 cells (Fig. 2a and Supplementary Fig. 2a). Whereas co-expression of FAM126A-N with EFR3B, the membrane anchor for the PI4KIIIα–EFR3B heterodimer, did not change the FAM126A-N localization (Fig. 2b, bottom), co-expression of FAM126A-N or full-length FAM126A with both EFR3B and TTC7B resulted in a relocalization of FAM126A to the plasma membrane (Fig. 2c and Supplementary Fig. 2b). Further, co-expression of FAM126A-N, TTC7B, EFR3B and PI4KIIIα resulted in co-localization of all four proteins at the plasma membrane in a manner that was dependent on the presence of TTC7B (Fig. 2d,e). Omission of EFR3B revealed a cytosolic co-localization (but nuclear exclusion) of TTC7B, FAM126A-N and PI4KIIIα (Fig. 2f). Collectively, these data argue for a central role of TTC7B not only in bridging PI4KIIIα to EFR3B, the plasma membrane anchor, as reported previously, but also in mediating the association of FAM126A to the plasma membrane, consistent with a direct interaction.

The localization experiments did not, however, allow us to conclusively determine whether, at the plasma membrane, FAM126A and PI4KIIIα can bind to TTC7B simultaneously, or, alternatively, whether FAM126A competes with PI4KIIIα for binding to TTC7B. To address this issue, we first confirmed the direct interaction between FAM126A-N and TTC7B by co-expression of the proteins in Escherichia coli and analysis of the resulting complex by size-exclusion chromatography (Fig. 3a). The two proteins co-eluted as a heterodimer. Importantly, protein–protein interaction experiments with purified proteins showed that the TTC7B/FAM126A-N subcomplex interacts directly with PI4KIIIα (Fig. 3b), ruling out a competition between FAM126A and PI4KIIIα for TTC7 binding.

Given that the TTC7B/FAM126A-N heterodimer bound tightly to PI4KIIIα, we next examined the effects of each of these proteins on the catalytic activity of PI4KIIIα. We purified wild-type (WT) or kinase-dead PI4KIIIα alone or in complex with either TTC7B alone or both TTC7B and FAM126A-N (Supplementary Fig. 3a) and assessed the relative kinase activity in vitro by monitoring the formation of PtdIns(4)P from PtdIns in the presence of γ32P-labelled ATP. The PI4KIIIα/TTC7 complex was about twice as enzymatically active as PI4KIIIα alone, and the PI4KIIIα/TTC7B/FAM126A-N ternary complex was roughly fivefold more active (Fig. 3c). Thus, both TTC7B and FAM126A seem to play a role in stabilizing the PI4KIIIα fold and/or stimulating the intrinsic enzymatic activity of the kinase.

To obtain further insights into the role of FAM126A in the kinase complex, we determined the crystal structure of the TTC7B/FAM126A-N dimer at 2.9 Å resolution (Fig. 4a and Supplementary Fig. 3b). Both proteins are almost entirely α-helical. In TTC7B, as in its yeast homologue Ypp1 (ref. 20; PDBID 4N5C), the alpha helices are arranged into a superhelix, with the
increases in the levels of PI4KIIIα decreases in the levels of PI4KIIIα in these cells. Indeed, immunoblot analysis revealed and 6), consistent with the misfolding and near-complete degradation the FAM126A protein (Fig. 5 a, lanes 3, 5 and 7), and cells from nonsense or missense mutations in FAM126A residues, and we propose that these surfaces together form surface comprising the C-terminal portion of TTC7B and adjoining that in the TTC7B/FAM126A-N subcomplex there is a conserved mammalian TTC7 interacts with PI4KIIIα, we reason that PI4KIIIα system present only in higher eukaryotes. Nevertheless, the overall organization of EFR3, TTC7 and PI4KIIIα within the complex is most likely conserved (Fig. 4d). Thus, as the yeast homologue of TTC7 interacts with the kinase through conserved surfaces in the C-terminal lobe, we reason that mammalian TTC7 interacts with PI4KIIIα in a similar way. We note that in the TTC7B/FAM126A-N subcomplex there is a conserved surface comprising the C-terminal portion of TTC7B and adjoining FAM126A residues, and we propose that these surfaces together form the binding site for PI4KIIIα in mammals (Fig. 4c).

The structural data suggest a stabilizing function for FAM126A in the PI4KIIIα complex. To test this hypothesis, we first examined the levels of PI4KIIIα complex components in primary human skin fibroblasts from five HCC patients that are homozygous for either nonsense or missense mutations in FAM126A (refs 3,21,23). Cells from patients with nonsense mutations were completely devoid of the FAM126A protein (Fig. 5a, lanes 3, 5 and 7), and cells from patients with missense mutations (L33P and C57R, see Supplementary Fig. 3c) exhibited greatly reduced levels of FAM126A (Fig. 5a, lanes 4 and 6), consistent with the misfolding and near-complete degradation of FAM126A in these cells. Indeed, immunoblot analysis revealed decreases in the levels of PI4KIIIα and its adaptors TTC7A, TTC7B and EFR3A (EFR3B was not detectable in the fibroblasts; Fig. 5a and Supplementary Fig. 4a), suggesting a general destabilization and degradation of the PI4KIIIα complex components in the absence of FAM126A. Accordingly, reintroduction of GFP-tagged FAM126A into the patient fibroblasts using a lentiviral vector partially rescued this phenotype (Supplementary Fig. 4b). Although a compensatory increase in FAM126B, a paralogue of FAM126A, was also observed in patient fibroblasts (Fig. 5a and Supplementary Fig. 4a), the approximately tenfold lower expression of FAM126B messenger RNA in fibroblasts (Supplementary Fig. 4c) may explain why this increase is not sufficient to compensate for lack of FAM126A.

High-performance liquid chromatography (HPLC) analysis of total phosphoinositide content revealed a decrease in total cellular PtdIns(4)P in HCC fibroblasts relative to control fibroblasts (Fig. 5b), and a specific decrease of the plasma membrane fraction of PtdIns(4)P in HCC fibroblasts relative to control fibroblasts was confirmed by immunofluorescence (Fig. 5c and Supplementary Fig. 4d). Thus, FAM126A loss specifically affects PI4KIIIα complex assembly and PI4KIIIα-mediated PtdIns(4)P synthesis at the plasma membrane.

Global KO of PI4KIIIα in several model organisms leads to lethality, whereas loss of FAM126A is permissive of life, although it causes defects in myelination in humans, leading to HCC (ref. 3). To investigate the potential role of FAM126A in promoting PI4KIIIα function in myelination, we compared the expression levels of PI4KIIIα complex subunits in mouse primary cortical neurons and oligodendrocytes. We found that FAM126A was expressed in both oligodendrocytes and neurons, whereas its paralogue FAM126B was expressed at much lower levels in oligodendrocytes relative to neurons (Fig. 5d and Supplementary Fig. 4a). Thus, in principle, a global loss of FAM126A could, in the brain, be more effectively compensated by FAM126B in neurons than in oligodendrocytes and thus more severely affect PI4KIIIα function in the latter cell type.

To support this possibility, we evaluated the levels of PI4KIIIα complex components in brain tissue from FAM126A KO mice. Although these mice do not exhibit an obvious abnormal phenotype...
or specific defects in myelination as evaluated by light and electron microscopy analysis (Supplementary Fig. 5), immunoblot analysis of lysates from total brain and optic nerve (a pure white matter tract) revealed a selective decrease of TTC7A and EFR3A relative to WT (Fig. 5e,f and Supplementary Fig. 4a). These are the isoforms of TTC7 and EFR3 whose levels are more highly expressed in oligodendrocytes relative to neurons, compared with their respective paralogues TTC7B and EFR3B (Fig. 5d and Supplementary Fig. 4a).

These results are further supported by immunoblot analysis of cellular fractions of different lineages that were generated from dissociated brain tissue by affinity purification from WT and FAM126A KO mice. Notably, there was a more pronounced decrease in PI4KIIIα, EFR3A/B and TTC7A/B in cells of the oligodendroglial lineage compared with those of the neuronal lineage (Supplementary Fig. 4e). Taken together, these data connect FAM126A to PI4KIIIα function in the nervous system and implicate the white matter as a region potentially susceptible to PI4KIIIα dysfunction following global loss of FAM126A, pointing to a mechanistic hypothesis for HCC disease pathogenesis. Although it seems surprising that the lack of FAM126A in mice does not produce the neurological phenotype observed in humans, we note that the greater diameter of axons and thickness of the myelin sheath around such axons in humans implies a larger surface area of oligodendrocytes. Thus, a greater demand on the machinery responsible for myelin biogenesis and maintenance in humans may explain their enhanced sensitivity to loss of FAM126A and impaired PtdIns(4)P synthesis.

Interestingly, FAM126A expression was reported to be repressed by activated β-catenin, and canonical Wnt/β-catenin/TCF signalling was recently shown to be a powerful negative regulator of oligodendrocyte differentiation, a step that precedes the massive plasma membrane biogenesis that occurs in myelination. Our data suggest that activation of PI4KIIIα may be an important downstream consequence of the relief of Wnt/β-catenin/TCF signalling that occurs in oligodendrocyte development.

In sum, our results demonstrate that FAM126A (hyccin) is an intrinsic component of the PI4KIIIα complex and an

Figure 4 Crystal structure of a TTC7B/FAM126A co-complex reveals an unusually large protein–protein interface and a conserved binding surface for PI4KIIIα. (a) Ribbon diagram for the TTC7B/FAM126A-N complex. Note the FAM126A-N hairpin structure that wraps around TTC7B like an arm. Point mutations in FAM126A that underlie HCC are indicated in Supplementary Fig. 3c. (b) Ribbon diagrams for TTC7B and FAM126A-N coloured from blue (N terminus) to red (C terminus). The ‘arm’ in FAM126A is green. Disordered residues absent from the model are indicated by dotted lines. (c) Space-filling models of the TTC7B/FAM126A-N complex (left) and TTC7B (middle and right) coloured by sequence conservation. Conserved surfaces on TTC7B/FAM126A-N (yellow outline) or TTC7B alone (orange outline) that may interact with PI4KIIIα are circled (left). The TTC7B surfaces at the interface with FAM126A-N are indicated by dotted yellow lines (middle and right). (d) Model for PI4KIIIα assembly at the plasma membrane. The EFR3 model is based on the structure of yeast Efr3 (ref. 20) (PDBID 4N5A).
important regulator of PtdIns(4)P production at the plasma membrane, the first step in the synthesis of the bulk of the downstream phosphoinositides PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. We further provide evidence for an important role of FAM126A in oligodendrocytes, a cell type whose specialized function is to help drive myelin growth^{10,11}. Collectively, our studies point to impaired production of these phosphoinositides as a mechanism through which absence of FAM126A results in a hypomyelinating leukoencephalopathy in patients, and they demonstrate the critical importance of plasma membrane phosphoinositide homeostasis in myelin development and potentially^{14} also in remyelination after myelin loss.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

J.M.B., X.W., R.C., T.C.W., K.M.R. and P.D.C. designed the experiments. J.M.B., X.W., R.C., M.S.O., C.M.S., M.M. and A.R. performed the experiments. E.G., S.A., S.B., R.B., E.Z., C.M. and M.S. generated and contributed cells, other tools, and reagents. J.M.B., K.M.R. and P.D.C. wrote the manuscript, with input from all authors.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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**METHODS**

**Plasmids and cloning.** TTC7B-mCherry, mCherry-P4KIIIt (NCBI NP_477352.3) and EFR3B-PA were previously described, and PM-mCherry was obtained from the De Camilli laboratory. 3xFLAG-PAKIIIt was generated by subcloning the FLAG-CMV-10 vector (Sigma) into the pEGFP-N1 vector (Clontech) using XhoI and EcoRI. TTC7B-3xFLAG was generated by subducing TTC7B into the p3xFLAG-CMV-14 vector (Sigma) using HindIII and XbaI. EFR3B-BFP and TTC7B-BFP were generated by subducing EFR3B into the pBAG-FP vector (Evrogen) using XhoI and EcoRI. TTC7B-3xFLAG was generated by subducing TTC7B into the pCMV-MYC-N vector (Clontech) using XhoI and NotI. TTC7B was generated by subducing TTC7B into the pCDNA5/FRT vector (Thermo Fisher) using NheI and NotI. 293T cell lines, TTC7BGFP, FAM126AGFP and GFP were subcloned into the pEGFP-C1 vector (Clontech) using KpnI and BamHI. For generation of stable HEK 293T cell lines, TTC7B-GFP, FAM126A-GFP and GFP were subcloned into the pCDNA5/FRT or pCDNA5/FRT/TO vectors (Thermo Fisher) using NheI and NotI. Transfection. Hela and HEK 293T cells were transfected with appropriate plasmids using Eugene HD (Promega) according to the manufacturer's instructions approximately 18-24 h before analysis. Exp293 cells were transfected with ExpFectamine (Thermo Fisher) according to the manufacturer's instructions.

**Immunoprecipitation and immunoblot.** HEK 293T cells stably expressing TTC7B-GFP, FAM126A-N-GFP or GFP were collected, resuspended in lysis buffer (150 mM NaCl, 20 mM Tris, 1 mM EDTA, 1% Triton X-100, pH 7.4, supplemented with protease inhibitors (Complete, EDTA-free (Roche)), sonicated and centrifuged for 10 min at 16,000 g. The supernatant was immunoprecipitated in lysis buffer by addition of GFP-trap agarose (Chromotek) and rocking for 1 h at 4°C. The resin was then isolated by centrifugation at 1,000 g, rinsed three times with lysis buffer, and analysed either by SDS-PAGE, immunoblot (with detection by chemiluminescence), or mass spectrometry (see below). Original (unoccupied) immunoblots are shown in Supplementary Fig. 6.

**Pulldown analysis by MS.** Peptides containing purified immunoprecipitates were rinsed three times with wash buffer (150 mM NaCl, 50 mM Tris, pH 7.4) and then desorbed for 30 min with urea (8 M) in 0.1 M Tris, pH 7.4, 1 mM dithiothreitol after alkylation and pre-digestion with endoproteinase LysC (Wako Chemicals USA). After incubation for 3 h samples were diluted fourfold with ammonium bicarbonate (25 mM) and further digested with trypsin (Promega) overnight. Digestions were stopped by addition of trifluoroacetic acid (TFA, 1 μl) and the resulting peptides were loaded and desalted on C18 Stage Tips.

**LC-MS/MS analysis.** Peptides were eluted from C18 Stage Tips with 60 μl of elution buffer (80% acetonitrile and 0.1% formic acid) and samples were dried down to 5 μl in a vacuum centrifuge. Peptides were then subjected to reverse phase chromatography on an Easy nLC 1000 system (Thermo Fisher Scientific) using a 15-cm column (New Objective) with an inner diameter of 75 μm, packed in-house with 1.9 μm C18 resin (Dr. Maisch). Peptides were eluted with an acetonitrile gradient (5–30% for 95 min at a constant flow rate of 250 nl min⁻¹) and directly electrosprayed into a mass spectrometer (Q Exactive; Thermo Fisher Scientific). Mass spectra were acquired on the spectrometer in a data-dependent mode to automatically switch between full-scan MS and up to 10 data-dependent MS/MS scans. The maximum injection time for full scans was 20 ms, with a target value of 3,000,000 at a resolution of 70,000 at m/z = 200. The ten most intense multiple charged ions (z ≥ 2) from the survey scan were selected with an isolation width of 3 Th and fragmented with higher-energy collision dissociation (HCD) with normalized collision energies of 25. Target values for MS/MS were set to 1,000,000 with a maximum injection time of 120 ms at a resolution of 17,500 at m/z = 200. To avoid repetitive sequencing, the dynamic exclusion of sequenced peptides was set to 20 s.

**MS data analysis.** MS and MS/MS spectra were analysed using MaxQuant (version 1.4.0.5), using its integrated ANDROMEDA search algorithms. Scoring of peptides for identification was carried out with an initial allowed mass deviation of the precursor ion of up to 6 ppm for the search for peptides with a minimum length of six amino acids. The allowed fragment mass deviation was 20 ppm. The false discovery rate (FDR) was set to 0.01 for proteins and peptides. Peak lists were searched against a local database for human proteome. Maximum missed cleavages were set to 2. The search included carbamidomethylation of cysteines as a fixed modification and methionine oxidation and N-terminal acetylation as variable modifications. All calculations and plots were performed as described previously using the R software package.

**Imaging.** For live-cell imaging, cells were grown in glass-bottom dishes (no. 1.5 thickness, MatTek Corporation). For immunofluorescence, cells were grown on coverslips (no. 1 thickness, neuVitro). Immunofluorescence labelling for PfDNA(4)P was performed as previously described. Imaging experiments were performed on a spinning-disc confocal microscope, using the PerkinElmer UltraView VoX system including a Nikon Ti-E Eclipse inverted microscope equipped with Perfect Focus, a temperature-controlled stage, a 14-bit electron-multiplying CCD (charge-coupled device) camera (Hamamatsu C9100-50), and a spinning-disc confocal scan head (CSU-X1, Yokogawa) controlled by Volocity software (PerkinElmer). All images were acquired through a 60× or 100× oil objective (1.4 NA, CFI Plan Apo VC). Blue fluorescence was excited with a 405 nm/50 mW diode laser (Melles Griot) and collected by a BP 445/60 filter. Green fluorescence was excited with a 488 nm/50 mW diode laser (Coherent) and collected by a BP 527/55 filter. Red fluorescence was excited with a 561 nm/50 mW diode laser (Coherent) and collected by a BP 590/50 filter. Multicolour images were acquired sequentially. Image acquisition was performed using the Volocity software (PerkinElmer) and image analysis was performed using Fiji for the quantification of plasma membrane PfDNA(4)P immunofluorescence. Samples were blinded, and average intensity...
**Methods**

**Protein expression (bacterial).** Plasmids containing the coding sequences for His-SUMO-TCCTB\(_{\text{dimer}}\) and GST-FAM126A\(_{\text{dimer}}\) were co-transformed into E. coli BL21(DE3) cells. The cells were grown to an OD\(_{600}\) of 0.7 at 37°C and then shifted to 18°C. Protein expression was induced at 18°C by addition of isopropyl-\(\beta\)-galactosidase (IPTG) to a final concentration of 0.5 mM. The cells were collected 20 h after induction and frozen at –80°C.

**Protein purification.** For the TTC7B\(_{\text{1-208}}\)-FAM126A\(_{\text{364–426}}\) complex, cells were resuspended in lysis buffer (20 mM Tris (pH 8.0 at 22°C), 200 mM NaCl, 1 mM Tris(2-carboxyethyl)phosphine (TCEP), and 20 mM imidazole) supplemented with protease inhibitors (Complete, EDTA-free (Roche)), and lysed using a cell disruptor (Avestin). The complex was first isolated using Ni-NTA resin (QIAGEN). After washing, the bound complex was eluted using 200 mM imidazole. Protein was further purified by size-exclusion chromatography on Superdex 200 (GE Healthcare) in buffer (20 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM TCEP). Selenomethionine-substituted proteins for structure determination were prepared similarly to native proteins as previously described.15

**Crystallization.** Crystals of native and selenomethionine-substituted TCCTB\(_{\text{dimer}}\)/FAM126A\(_{\text{dimer}}\) complexes were grown by the hanging-drop method at 20°C, mixing 1.35 μl each of protein solution (6 mg ml\(^{-1}\)) and mother liquor (0.1 M Hepes (pH 7.0–7.4), 4–5% polyethylene glycol (PEG) 8000) and 0.3 μl of 0.2 M 3-[(1-pyrindino)-1-propane sulfonate (NDSB-201). The crystals belong to space group P2\(_1\)2\(_1\)2\(_1\), with two copies of the complex in the asymmetric unit.

**Data collection and structure determination.** Crystals were serially transferred into mother liquor supplemented with ethylene glycol from 10 to 30%, loop mounted, and flash-frozen in liquid nitrogen. Diffraction data were collected at the selenium anomalous edge. For both data collection and selenomethionine-substituted crystals were collected at the NE-CAT beamline 24ID-E at the Advanced Photon Source (APS). The data were processed using HKL2000 (ref. 36; Supplementary Table 2). For phasing, we used the selenomethionine-substituted crystals in the SAD method.16 Phasing was carried out using PHENIX (ref. 38). A representative electron density map is shown in Supplementary Fig. 3b (top). We built the model of the complex using COOT (ref. 39) and refined it against native data in PHENIX (ref. 38) by positional, translation/libration/scroll motion, and individual B-factor refinement options and secondary-structure restraints (Supplementary Table 2 and Supplementary Fig. 3b (bottom)). The final structure has good geometry (98.3 and 1.7% of residues are in allowed and generally allowed regions of the Ramachandran plot, respectively). The N terminus of one of the two TCCTB\(_{\text{dimer}}\)-FAM126A\(_{\text{dimer}}\) asymmetric units is not ordered and was not modeled. Several loops were omitted in both copies of TCCTB (A: residues 33–36, 157–166, 197–212, 292–299, 343–358, 462–464, 619–686; B: 9–139, 154–170, 194–213, 286–318, 342–362, 460–464, 618–688) and FAM126A (A: residues 2–7, 18–23, 30–33, 93–94, 149–153, 288–308; B: 2–6, 16–24, 30–35, 49–52, 93–98, 114–120, 140–157, 288–308).

**Protein expression (mammalian) for pulldown and kinase assay.** Expi293 cells (Thermo Fisher) were maintained and transfected with ExpiFectamine (Thermo Fisher) according to the manufacturer’s instructions. Seventy-two hours after transfection, the viral supernatant was collected, filtered through a 0.45 μm filter, and centrifuged in ultraviolet-sterilized tubes at 37693g for 90 min at 4°C using an SW28.1 rotor (Beckman). The liquid was decanted, and 250 μl of OptiMEM (Thermo Fisher) was added to the tube and incubated at 4°C overnight. The following day, the virus was gently resuspended, virus titre was measured by ELISA (p24 HIV ELISA kit, Cell Lysis Assay kit) as previously described.17 The E2 offering created in the mixed 129/5C7BL6 genetic background were backcrossed for ten generations into the C57BL/6 pure strain. Animal care and use was carried out in accordance with institutional guidelines (Animal Care and Use Committee of Istituto di Fisiologia Clinica, CNR, Pisa, Italy). Primary cortical neuronal and oligodendroglial cultures were generated as previously described.18 Samples used for immunoblot analysis (Fig. 5) were collected at DIV16 (neurons) and DIV5 (oligodendrocytes). No statistical method was used to predetermine samples size, and the experiments were not randomized.

**Lentivirus production and rescue experiment.** Lentiviral particles were purified as described herein. HEK 293T cells were transfected with either pL3.7-FAM126A\(_{\text{dimer}}\)-GFP or pL3.7 empty vector in combination with the packaging plasmids pMD2.G and psPAX2 using Lipofectamine 2000 (Thermo Fisher) according to the manufacturer’s instructions: Seventy-two hours after transfection, the viral supernatant was collected, filtered through a 0.45 μm filter, and centrifuged in ultraviolet-sterilized tubes at 37693g for 90 min at 4°C using an SW28.1 rotor (Beckman). The liquid was decanted, and 250 μl of OptiMEM (Thermo Fisher) was added to the tube and incubated at 4°C overnight. The following day, the virus was gently resuspended, virus titre was measured by ELISA (p24 HIV ELISA kit, Cell Lysis Assay kit) as previously described.17 The E2 offering created in the mixed 129/5C7BL6 genetic background were backcrossed for ten generations into the C57BL/6 pure strain. Animal care and use was carried out in accordance with institutional guidelines (Animal Care and Use Committee of Istituto di Fisiologia Clinica, CNR, Pisa, Italy). Primary cortical neuronal and oligodendroglial cultures were generated as previously described.18 Samples used for immunoblot analysis (Fig. 5) were collected at DIV16 (neurons) and DIV5 (oligodendrocytes). No statistical method was used to predetermine samples size, and the experiments were not randomized.

**Quantitative RT–PCR analysis.** Total RNA was isolated from control primary human skin fibroblasts using the RNeasy kit (Thermo Fisher) according to the manufacturer’s instructions and stored in nuclease-free water at –20°C. Total RNA (2 μg) was reverse transcribed with an oligo(dT) primer using SuperScript polymerase (Thermo Fisher). cDNA was analysed in triplicate by qRT–PCR amplification using SYBR Green Maxima on a Bio-Rad CFX96 Real-Time PCR Detection System. PCR amplification conditions were as follows: 95°C (2 min) and 4 cycles of 95°C (5 s) and 60°C (30 s). Primer pairs (FAM126A: 5'-CAGACAGTGCGTTCTGCTG-3' and 5'-TCTCCACACACACACCTCTC-3'; FAM126B: 5'-TCCCTCTTATCAGGCT-3' and 5'-ATGTCGACGACCTCCCTT-3') were designed to amplify mRNA-specific fragments, and unique products were tested by melt-curve analysis. PCR efficiencies were 101.6% (slope of –3.285 in a tenfold dilution series) for FAM126A and 96.7% (slope of –3.404 in a tenfold dilution series) for FAM126B using the indicated primers. Data were analysed using ΔΔC\(_T\), and values were normalized to the housekeeping gene ribosomal protein S26 (using 5'-CCTGCGTCCAGATGCAA-3' and 5'-GCAATGCGAATTTCTTAAAGCG-3' as primers).

**Immunosolation of oligodendrocyte and neuronal lineage cells.** For acute cell isolation, cells were isolated using the MACS cell selection kit (Miltenyi Biotech). Briefly, brains were from male FAM126A KO and littermate WT control mice (on the pure C57BL/6 background) at postnatal day 8 were isolated, minced, and mechanically dissociated with a gentleMACS dissociator according to the manufacturer’s instructions. The cells were then passed through a 45-μm strainer.
and incubated with the cell-specific beads for 15 min. The cells were then loaded on LS columns and separated on a quadroMACS magnet, first with anti-O4 beads (elution was labelled oligodendrocyte lineage), and then with anti-ACSA beads to deplete astrocytes, and the remainder (flow-through) was a sample enriched in cells of the neuronal lineage. The target cells were washed once in PBS, then pelleted and snap-frozen. Pellets were subsequently thawed, and lysates were generated for immunoblot analysis.

**Light and electron microscopic imaging of myelin in FAM126A KO mice.** Male mice at age P15 (three FAM126A KO and two littermate WT controls, on a mixed 129/C57BL/6 background) were perfused transcardially with 2% paraformaldehyde, 2.5% glutaraldehyde in 100 mM cacodylate buffer pH 7.4. Samples were postfixed with 1% osmium tetroxide, 1.5% potassium ferrocyanide in 100 mM cacodylate buffer pH 7.4, en bloc stained with uranyl acetate, dehydrated in increasing concentrations of ethanol and propylene oxide, and finally embedded in Epon. Samples were cured at 60°C in an oven for 48 h. Epon blocks were sectioned using a Leica EM UC7 ultramicrotome (Leica Microsystems). For light microscopy, semithin sections (1 µm) of optic nerve, corpus callosum (sagittal section of the central area), and spinal cord (ventral funiculus cervical region) were generated and stained with toluidine blue. Light microscopy was performed on a Zeiss Axio Imager equipped with a Plan-Apochromat 100×/1.4 oil objective with an AxioCam MRC 5 colour CCD camera. For electron microscopy, ultrathin sections (60 nm) were contrasted with 2% uranyl acetate and Sato’s lead solutions and observed with a LEO 912AB Zeiss Transmission Electron Microscope (Carl Zeiss). Digital micrographs were taken with a 2k × 2k bottom-mounted slow-scan Proscan camera (ProScan) controlled by the EsivisionPro 3.2 software (Soft Imaging System).

**Statistics and reproducibility.** Statistical analysis was performed using either Microsoft Excel or Prism 6 software. Detailed statistical information (which statistical test was used, number of independent experiments, P values, definition of error bars) is listed in individual figure legends. All immunoblots were repeated at least three times except for some immunoblots shown in Fig. 5, which were repeated twice (see legend for Supplementary Fig. 4a for details). All imaging experiments were repeated three times. No statistical method was used to predetermine samples size for animal experiments, and the experiments were not randomized.

**Accession numbers.** Structure factors and coordinates for the TTC7/FAM126A-N complex have been deposited in the Protein Data Bank (accession number 5DSE).

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