B56δ-related protein phosphatase 2A dysfunction identified in patients with intellectual disability

Gunnar Houge,1,2 Dorien Haesen,3 Lisenka E.L.M. Vissers,4 Sarju Mehta,5 Michael J. Parker,6 Michael Wright,7 Julie Vogt,8 Shane McKee,9 John L. Tolmie,10 Nuno Cordeiro,11 Tjitske Kleefstra,12 Marjolein H. Willemsen,4 Margot R.F. Reijnders,4 Siren Berland,8 Eli Hayman,12 Eli Lahat,9 Eva H. Brilstra,13 Koen L.I. van Gassen,13 Evelien Zonneveld-Huijssoon,13 Charlotte I. de Bie,14 Alexander Hoischen,8,15 Evan E. Eichler,14 Rita Holdhus,2 Vidar M. Steen,1,2 Stein Ove Doskeland,15 Matthew E. Hurles,16 David R. FitzPatrick,17 the Deciphering Developmental Disorders (DDD) study,18 and Veerle Janssens3

1Center for Medical Genetics and Molecular Medicine, Haukeland University Hospital, Bergen, Norway. 2Department of Clinical Science, University of Bergen, Bergen, Norway. 3Laboratory of Protein Phosphorylation and Proteomics, Department of Cellular and Molecular Medicine, KU Leuven, University of Leuven, Leuven, Belgium. 4Department of Human Genetics, Radboud Institute for Molecular Life Sciences and Donders Centre for Neuroscience, Radboud University Medical Center (RUMC), Nijmegen, Netherlands. 5East Anglian Medical Genetics Service, Addenbrookes Hospital, Cambridge, United Kingdom. 6Sheffield Clinical Genetics Service, Sheffield Children’s Hospital, Sheffield, United Kingdom. 7Northern Genetics Service, Newcastle upon Tyne Hospitals, Newcastle upon Tyne, United Kingdom. 8West Midlands Regional Genetics Service, Birmingham Women’s Hospital, Birmingham, United Kingdom. 9Northern Ireland Regional Genetics Centre, Belfast City Hospital, Belfast, United Kingdom. 10West Scotland Genetics Services, Southern General Hospital, NHS Greater Glasgow and Clyde, Glasgow, United Kingdom. 11Children’s Services - NHS Ayrshire and Arran, Rainbow House, Ayrshire Central Hospital, United Kingdom. 12Pediatric Neurology Department, Asaf Harofeh Medical Center, Zrifin, Israel. 13Department of Medical Genetics, UMC Utrecht, Utrecht, Netherlands. 14Department of Genome Sciences, University of Washington, Seattle, Washington, USA. 15Department of Biomedicine, University of Bergen, Bergen, Norway. 16Wellcome Trust Sanger Institute, Genome Campus, Hinxton, Cambridge, United Kingdom. 17MRC Human Genetics Unit, MRC Institute of Medical Genetic and Molecular Medicine, Edinburgh, United Kingdom. 18DDD project (members detailed in supplemental material), Wellcome Trust Sanger Institute, Cambridge, United Kingdom.

Here we report inherited dysregulation of protein phosphatase activity as a cause of intellectual disability (ID). De novo missense mutations in 2 subunits of serine/threonine (Ser/Thr) protein phosphatase 2A (PP2A) were identified in 16 individuals with mild to severe ID, long-lasting hypotonia, epileptic susceptibility, frontal bossing, mild hypertelorism, and downslanting palpebral fissures. PP2A comprises catalytic (C), scaffolding (A), and regulatory (B) subunits that determine subcellular anchoring, substrate specificity, and physiological function. Ten patients had mutations within a highly conserved acidic loop of the PPP2R5D-encoded B56δ regulatory subunit, with the same E198K mutation present in 6 individuals. Five patients had mutations in the PPP2R1A-encoded scaffolding Aα subunit, with the same R182W mutation in 3 individuals. Some Aα cases presented with large ventricles, causing macrocephaly and hydrocephalus suspicion, and all cases exhibited partial or complete corpus callosum agenesis. Functional evaluation revealed that mutant A and B subunits were stable and uncoupled from phosphatase activity. Mutant B56δ was A and C binding–deficient, while mutant Aα subunits bound B56δ well but were unable to bind C or bound a catalytically impaired C, suggesting a dominant-negative effect where mutant subunits hinder dephosphorylation of B56δ-anchored substrates. Moreover, mutant subunit overexpression resulted in hyperphosphorylation of GSK3β, a B56δ-regulated substrate. This effect was in line with clinical observations, supporting a correlation between the ID degree and biochemical disturbance.

Introduction
Unlike protein kinases, mutations in serine/threonine (Ser/Thr) protein phosphatases have not commonly been associated with disorders of human development. There are 2 major Ser/Thr protein phosphatase families in the cell: protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), together accounting for more than 90% of all phospho-Ser/Thr dephosphorylations. PP2A consists of a catalytic subunit (C), a substrate binding regulatory subunit (B), and a scaffolding subunit (A) that links B and C. Unlike the generally expressed A and C subunits, there is a plethora of B subunits with different expression patterns (1). The differential substrate preferences of the nearly 100 different PP2A holoenzymes that, in theory, can be formed by 2 C isoforms, 2 A isoforms, and at least 23 types of B subunits is largely unknown (2), particularly within the context of a whole organism. Apparently, PP2A-dependent protein dephosphorylation has a potential for regulation that may be just as fine-tuned as protein phosphorylation. Unlike protein phosphorylation, associations between mutations in PP2A subunits and genetic diseases or syndromes have not been described until recently, when 4 de novo PPP2R5D and 3 de novo PPP2R1A mutations were found among the first 1,133 parent-child trios sequenced in the United Kingdom Deciphering Developmental Disorders project (3).

Here, we add clinical descriptions and functional data to the DDD findings and present 9 additional cases with de novo PP2A subunit mutations; 7 in PPP2R5D, encoding the regulatory B56δ subunit, and 2 in PPP2R1A, encoding the scaffolding Aα subunit.
Taken together, of the 11 mutations in PPP2R5D, 6 mutations and 2 mutations were identical; 3 of the 5 mutations in PPP2R1A were also identical. All Aα mutations and all but one of the B56δ mutations had the potential to hinder access of catalytically competent C subunits to B56δ-regulated substrates, suggesting a common dominant-negative disease mechanism mainly affecting B56δ-regulated Ser/Thr dephosphorylation.

Results

In cases with intellectual disability (ID) of unknown etiology, parent-child trio exome sequencing was performed to find de novo and recessive mutations that could explain the condition. De novo missense mutations in 2 subunits of the Ser/Thr phosphatase PP2A were identified in 16 individuals from the United Kingdom (7 cases), the Netherlands (7 cases), Israel (1 case), and Norway (1 case).

The 7 United Kingdom cases were found among 1,133 chromosomally normal parent-child trios (3). This suggests that the prevalence of PP2A subunit mutations in the moderate-to-severe ID group without pathogenic copy number aberrations is around 0.6%. In the United Kingdom, this was part of the large DDD project (http://www.ddduk.org); in other cases, this was done as part of routine diagnostics. In 11 cases, de novo missense mutations in PPP2R5D, encoding the regulatory B56δ PP2A subunit, were found. In 5 other cases, a de novo missense mutation in PPP2R1A, encoding the scaffolding Aα subunit of PP2A, was found. Six mutations and 2 mutations in PPP2R5D were identical, and 3 PPP2R1A mutations were identical. Details on all mutations can be found in Table 1. Other trio exome sequencing results indicating a de novo change of possible relevance or a recessive condition of potential interest can be found in Supplemental Table 1 (supplemental material available online with this article; doi:10.1172/JCI79860DS1). In 10 cases, such findings were made, but based on bioinformatic evaluation of the variants and the clinical features of the patients, all but one of these findings could easily be excluded as causative factors for the phenotype. The exception was case 15, which had heterozygosity for a TMEM67 splice mutation and a few signs that were compatible with a ciliopathy (e.g., unilateral postaxial polydactyly). However, this could also be a random finding, since a second TMEM67 mutation was not found upon Sanger sequencing. In addition, detecting the same de novo missense mutations in patients with identical clinical features is, in itself, evidence in support of causality, especially when supported by functional data (see below). As a crude estimation, the likelihood of finding 10 de novo missense mutations in the same 9-amino acid stretch of B56δ by chance should be less than 10–60 (see Statistics).

The clinical features of the 11 PPP2R5D cases and the 5 PPP2R1A cases are summarized in Tables 2 and 3, respectively. Despite mutations occurring in 2 different PP2A subunit genes with different biochemical functions (regulatory and scaffolding), there are clinical similarities between the cases. All patients were born after a normal pregnancy, and 15/16 cases had birth weights within normal range. In 2 cases, breech deliveries were reported, and in 2 other cases, emergency cesarean sections had to be performed. After birth, ID and hypotonia were common features in all cases. Despite pronounced and long-lasting hypotonia, feeding difficulties were usually not a major problem, and only one case had gastrostomy. In 12/16 cases, the degree of ID was severe, and this correlated with very late independent walking, usually around age 6–7 years. The exceptions were the 4 patients with E200K, P201R, or W207R mutations (see below for functional explanation), who learned to walk between 1½ and 2½ years of age and had mild/moderate ID (Table 2). These 4 cases were also the only ones with language development beyond a few words. Seven out of 16 patients had epilepsy, including one of the mild ID cases. Only one patient had short stature (case 1 with a P53S mutation, see Table 2), and he was the only PPP2R5D case that was microcephalic. In the other PPP2R5D cases, head circumferences were from upper-normal range to pronounced macrocephaly, and in the latter cases, hydrocephalus was suspected. In contrast, most PPP2R1A cases were normocephalic or microcephalic, and hydrocephalus was initially suspected in only one case (Table 3). In all these patients, the corpus callosum was absent or almost absent, a feature that distinguished PPP2R1A cases from PPP2R5D cases. In contrast, facial features were overlapping (Figure 1): A hypotonic and sometimes also elongated face with tented upper lip, mild hypertelorism with downsloping palpebral fissures, and frontal bossing in the PPP2R5D cases.

The finding of recurrent and clustered de novo missense mutations in 2 PP2A subunit genes (PPP2R5D and PPP2R1A) suggested a dominant-negative– or gain-of-function–related disease mechanism, rather than haploinsufficiency or loss-of-function. All but one of the PPP2R5D mutations (E198K, E200K, P201R,
further strengthening our working hypothesis that a charge change in the acidic B56δ loop could be pathogenic.

Our cellular binding assays with Glutathione S-Transferase–tagged (GST-tagged) B subunits and HemAgglutinin-tagged (HA-tagged) WT or mutant Aα subunits revealed that all 3 PPP2R1A mutations also affected PP2A holoenzyme formation (Figure 3). Surprisingly, interaction with the C subunit was hindered, despite the Aα mutations being in HEAT domains predicted to interact with B (Figure 3A). The mutations’ effect on B subunit binding was complex (Figure 3B). All Aα mutants lacked significant binding to the B55α (also called B) family members tested (isoforms B55α and B55β), as well as to the B56δ (also called B’ or PR61) family members tested (B56α and B56γ). On the other hand, binding to B56γ was almost entirely retained, whereas B56ε bound significantly less. For PR72, a member of the B” family of PP2A regulatory subunits, binding was retained to Aα-P179L but was completely lost to the Aα-R182W and Aα-R258H mutants (Figure 3B). These (mutant) Aα binding characteristics were confirmed for endogenous B55α and B56δ subunits, for which good-quality, isoform-specific antibodies are available (Figure 4A). These data could be compatible with a dominant-negative effect on, notably, B56δ for all Aα mutants, and on PR72...
Table 2. Clinical features in cases with de novo PPP2R5D missense mutations

| Case | Mutation  | Age of examination | Sex | Delivery | Birth weight | Hypotonia | Walked unsupported | Ataxic gait | Language | Epilepsy | EEG/DD | ID/DD | Height | Head circumference | Weight | Brain MRI | Other findings |
|------|-----------|-------------------|-----|----------|-------------|-----------|-------------------|------------|----------|----------|--------|--------|--------|--------|----------------|--------|-----------|---------------|
| 1    | P53S      | 53 yr             | Male| Normal   | Normal      | Present   | Not reported      | -          | No       | No       | Abnormal| Severe | 0.5 cm | 1 cm × 3rd | 50th    | Normal | Cataract |
| 2    | E198K     | 5 yr              | Female| Breach  | Normal      | Present   | 6 yr              | Yes       | No       | No       | Abnormal| Severe | 10th   | 5 cm > 97th| 90th    | 50th    | Hydrocephalus |
| 3    | E198K     | 11 yr             | Female| Emergency C/S | Normal      | Present   | Not so far        | Yes       | No       | No       | Abnormal| Severe | 25th   | 2 cm < 3rd | 50th    | 90th    | Narrow palate |
| 4    | E198K     | 10 yr             | Male| Emergency C/S | Normal      | Present   | 7 yr              | Yes       | No       | No       | Abnormal| Severe | 50 cm  | 20 cm < 3rd| 90th    | 50th    | Mild ventricular dilatation |
| 5    | E198K     | 15 yr             | Male| Normal   | Normal      | Present   | 6 yr              | Yes       | No       | No       | Abnormal| Severe | 10th   | 20 cm < 3rd| 90th    | 50th    | Mild ventricular dilatation Small CC |
| 6    | E198K     | 13 yr             | Male| Normal   | Normal      | Present   | Not so far        | Yes, poor intelligence | No       | No       | No       | Abnormal| Severe | 5-10th | 1 cm > 97th| 90th    | 50th    | Mild |
| 7    | E198K     | 2 yr              | Female| Female | Normal      | Present   | 1½ yr             | No       | No       | No       | Abnormal| Severe | 50th   | 1 cm > 97th| 75th    | 50th    | Moderate |
| 8    | E200K     | 20 yr             | Female| Female | Normal      | Present   | 2 yr              | No       | No       | No       | Abnormal| Severe | 50th   | 5 cm > 97th| 75th    | 50th    | Moderate |
| 9    | E200K     | 4 yr              | Male| Female | Normal      | Present   | 1½ yr             | No       | No       | No       | Abnormal| Severe | 50th   | 1 cm > 97th| 75th    | 50th    | Normal |
| 10   | E200K     | 3 yr              | Male| Female | Normal      | Present   | 2 yr              | No       | No       | No       | Abnormal| Severe | 50th   | 1 cm > 97th| 75th    | 50th    | Normal |
| 11   | W201R     | 9 yr              | Female| Female | Normal      | Present   | 2 yr              | No       | No       | No       | Abnormal| Severe | 50th   | 1 cm > 97th| 75th    | 50th    | Normal |

Height, head circumference, and weight are measured relative to centiles (the 3rd and 97th centile correspond to ± 2 SD). Abbreviations: ID/DD, intellectual disability/developmental delay; y, years; -, unknown or not done; SGA, small for gestational age; C/S, cesarean section; CC, corpus callosum.

Discussion

The presented work demonstrates that de novo missense mutations in genes encoding PP2A subunits may cause syndromic ID — and
probably also nonsyndromic ID, since the facial dysmorphism in these cases is subtle (Figure 1). The **PPP2R5D** and **PPP2RIA** mutations disrupt B56δ-dependent dephosphorylation dynamics and link PP2A dysfunction to congenital brain dysfunction.

In general, the Aα cases were more severely affected than the B56δ cases. All had severe ID, absent speech, diminished brain growth, and partial or complete agenesis of the corpus callosum (Table 3). This is in line with the expected greater difficulty to compensate for a general scaffolding (A) subunit dysfunction than a specific regulatory (B) subunit dysfunction, as reflected by our biochemical data showing additional loss or reduction of holoenzyme assembly of many different PP2A complexes (B55α, B55β, B56α, B56γ, and B56δ) for these Aα mutants (Figure 3B). The Aα scaffolding subunit is highly flexible, composed of 15 tandem repeat HEAT motifs (11) that mediate interactions with a regulatory B subunit (HEAT repeats 1–8) and the C subunit (HEAT repeats 11–15)(4, 5, 12, 13). Two ID-associated **PPP2RIA** mutations (P179L and R182W) cluster in HEAT domain 5 of Aα, and one (R258H) occurs in HEAT domain 7; these mutations are associated with decreased specific activity of C.

Besides its scaffolding function, Aα is a major player in the biogenesis of active PP2A holoenzymes (14). This highly regulated but incompletely understood process does not only involve simple trimeric assembly of the A, B, and C subunits, but it also involves several activation steps of the C subunit, which is de novo translated as an inactive enzyme (15). It has been suggested that some of these activation steps require or are facilitated by the A subunit (16, 17), explaining why A-subunit mutations may affect the specific activity of the associated C subunit, as observed here within the B56δ-(mutant A)-C complexes (Figure 4C). Additional activity measurements performed directly in anti-HA immunoprecipitates of these activation steps require or are facilitated by the A subunit (16, 17), explaining why A-subunit mutations may affect the specific activity of the associated C subunit, as observed here within the B56δ-(mutant A)-C complexes (Figure 4C). Additional activity measurements performed directly in anti-HA immunoprecipitates (16, 17), explaining why A-subunit mutations may affect the specific activity of the associated C subunit, as observed here within the B56δ-(mutant A)-C complexes (Figure 4C).

**PPP2R5D** encodes the longest isoform of the B’ family of PP2A regulatory subunits and harbors unique N- and C-terminal extensions, which are predicted to be important for substrate recognition and/or subcellular targeting (18). Ten out of 11 **PPP2R5D** mutations were located in a conserved acidic loop of B56δ needed for holoenzyme formation (Table 1), and all mutations introduced a positively charged residue (either arginine or lysine). Only one mutation (P53S) was atypical, and this case also had a different clinical picture: it was the only **PPP2R5D** case with short stature and microcephaly (Table 2). In theory, P53S in the B56δ-specific N-terminal domain might change the PP2A-B56δ interaction with relevant substrates or introduce a new phosphorylation site that affects regulation by protein kinases. Such changes could easily have a gain-of-function or dominant-negative effect.

We also observed a correlation between the degree of biochemical disturbance and clinical severity. Among the **PPP2R5D** cases (Table 2), the 6 patients with E198K mutations were the most severely affected, in line with a near absence of A and C subunit binding. The least-affected individuals were the E200K cases, both with mild ID correlating with some residual A and C binding capability (Figure 2B). Notably, E198 is the only one of the 5 mutated residues that directly interacts with the catalytic subunit (Figure 2A and refs. 4–6). Additionally, since all B subunits,
except B56δ and PR72, have been shown to make stabilizing contacts with the C subunit tail (5, 19). The brain-restricted phenotype is not unexpected for B56δ mutations because PPP2R5D is expressed mainly in the brain, notably in the striatum (18, 20, 21). The brain-restricted phenotype may be related to our finding that mutated PPP2R5D is generally expressed as a common scaffolding subunit for many different PP2A holoenzymes (22).

A brain-restricted phenotype is not unexpected for B56δ mutations because PPP2R5D is expressed mainly in the brain, particularly in the striatum (18, 20, 21). The brain-restricted phenotype of the Aα mutations (P179L, R182W, and R258H) is more unexpected, since PPP2R1A is generally expressed as a common scaffolding subunit for many different PP2A holoenzymes (22). Nevertheless, other malformations than severe corpus callosum hypogenesis were not found in the 5 cases (Table 3). The restricted phenotype may be related to our finding that mutated PPP2R1A (Figure 3B), both of which are expressed in the brain, notably in the striatum where both B56δ and PR72 (encoded by PPP2R3A) control the dephosphorylation of the neural dopamine-regulated inhibitor of PP1 (DARPP-32) (23–25).

Despite the severe intellectual dysfunction in most patients, B56δ does not appear essential for mammalian brain development, since Ppp2r5δ knockout mice have intact learning and memory despite ataxia and tauopathy (21). This also suggests that our patients’ ID was not caused by haploinsufficiency or a mere loss of function. It is therefore tempting to speculate that the mutated B56δ subunits may not only interfere in a dominant manner with dephosphorylation of B56δ binding PP2A substrates, as shown for GSK-3β in HEK293 cells (Figure 5), but also with subcellular anchoring of PP2A via B56δ, and thereby with control of localized signaling. Thus, Aα-binding-deficient B56δ mutants may still form complexes with B56δ partners, but without promoting dephosphorylation. Such dysphosphorylation may have far-reaching consequences for regulation of localized signaling. One example could be the signaling complex scaffolded by the neural variant of the cAMP-dependent PKA anchoring protein mAKAP, that binds B56δ and several other phosphatases (PP1, PP2B) and kinases (PKA, PDK1, RSK3, ERK5) (21, 26). Dephosphorylation of PP2A-B56δ substrates may also be hindered, e.g., the transcription factor and PKA-substrate HAND (28), the nuclear cyclin-dependent kinase 5 (CDK5) activator CDK5R1 (21), and DARPP-32 (22, 25). PKA activates DARPP-32 directly by Thr-34 phosphorylation and indirectly by PP2A-B56δ-dependent activation through Thr-75 dephosphorylation (24). B56δ has several sites for PKA phosphorylation that activate PP2A-B56δ and indirectly by PP2A-B56δ-dependent activation through Thr-75 dephosphorylation (24). B56δ has several sites for PKA phosphorylation that activate PP2A-B56δ and indirectly by PP2A-B56δ-dependent activation through Thr-75 dephosphorylation (24). B56δ has several sites for PKA phosphorylation that activate PP2A-B56δ and indirectly by PP2A-B56δ-dependent activation through Thr-75 dephosphorylation (24). B56δ has several sites for PKA phosphorylation that activate PP2A-B56δ and indirectly by PP2A-B56δ-dependent activation through Thr-75 dephosphorylation (24).
**Figure 3. Binding of mutant and WT Aα to C and B subunits.** (A) PP2A-C subunit binding assays: HA-tagged WT Aα, 3 ID-associated Aα mutants (P179L, R182W, and R258H), or an empty HA-vector (-) were transfected into HEK293 cells. Following anti-HA immunoprecipitation, the presence of endogenous C subunit in the immunoprecipitates was examined by immunoblotting (IB). After quantification of the band intensities with ImageJ software, the ratios between HA and C signals were determined and calculated relative to WT Aα control. Mean values and a representative image of 3 independent experiments are shown (1-way multiple-comparisons ANOVA, **P < 0.01). (B) PP2A B subunit binding assays: Several GST-tagged B subunits, belonging to 3 different families (B55 or B, B56 or B′, and B′′) or GST alone (-) were coexpressed in HEK293 cells with HA-tagged WT Aα, or ID-associated Aα-P179L, R182W, and R258H mutants. The presence of HA-Aα (WT or mutant) in the complete lysates and the isolated GST pulldown complexes was determined by IB. After quantification of the band intensities with ImageJ software, the ratios between GST and HA signals were determined and calculated relative to WT Aα control (which were set to 100% for each B-type subunit pulldown). Mean values and a representative image of 3 independent experiments are shown (1-way multiple-comparisons ANOVA; *P < 0.05, **P < 0.01, ***P < 0.001).
For future evaluation of de novo mutation origin (paternal or maternal)(37), it is of interest that all 3 PPP2R1A mutations are also found in the Sanger Institute's Catalogue of Somatic Mutations in Cancer (the COSMIC database; http://cancer.sanger.ac.uk/cosmic), mainly in endometrial and ovarian cancers (38, 39). Aα P179L/P179R, R182W, and R258H are by far the most prevalent mutations. A growth advantage may also explain mutation recurrence if these de novo mutations turn out to be solely paternal (40). Since 88% of the cancer-associated Aα mutations are of the missense-variant, a dominant-negative effect also in cancer promotion is likely. None of our patients have been diagnosed with or treated for cancer. The cancer risk might not be increased, in line with what is usually the case for congenital gain-of-function mutations in other cancer-related pathways like the RAS/MAPK pathway or the PI3K/akt cascade. Only further patients and patient follow-ups will answer this question, but a major cancer risk seems unlikely.

The tumor-suppressor effect of PP2A may operate by KRAS/MAPK cascade inhibition, KRAS/ARF/TP53 cascade inhibition, or PI3K/akt/TP53 cascade inhibition (33). Somatic mosaic activation of the PI3K/akt cascade causes the megalencephaly-capillary malformation-polymicrogyria (MCAP) and megalencephaly-polymicrogyria-polydactyly-hydrocephalus (MPPH) group of overgrowth syndromes (41). These patients have variable ID, a tendency to develop hydrocephalus and epilepsy, and dysmorphic facial features, including frontal bossing with hypotonia, tented upper lip, and deep-set eyes. The latter features are shared with several of our patients (Figure 1). It is therefore relevant to consider B56δ-dependent PP2A dysregulation syndrome (which we propose to be designated B56δdeltopathies) among the differential diagnoses to the MCAP/MPPH group of syndromes, at least in some cases. It is conceivable that the B56δ mutations may affect only a subgroup of PP2A substrates located distally in the PI3K signaling cascade — such as OsK-3β Ser9, a well-established Akt phosphorylation site — since the proximal steps do not appear to be subject to B56δ-dependent dephosphorylation (42).

In summary, we have demonstrated that de novo missense mutations in the PPP2RSD and PPP2RIA genes encoding PP2A subunits represent a new mechanism for ID, due to disrupted B56δ-dependent dephosphorylation dynamics and PP2A dysfunction.

**Methods**

**Case detection.** There were 7 Dutch patients (from RUMC: cases 1, 8–9, and 11; from UMC Utrecht: cases 7, 15, and 16). Six of these cases were identified through routine diagnostic exome sequencing...
The de novo PPP2R5D mutations in the Norwegian (case 2) and Israeli (case 6) patients were identified by exome sequencing of parent-child trios in a diagnostic setting. Only the PPP2R5D variant remained as true de novo after filtering and verification by Sanger sequencing. Cases 1 and 11 have been previously published as part of studies showing the power and impact of next-generation sequencing–based (NGS-based) technologies in a clinical diagnostic setting without clinical details on the patients’ phenotypes or functional evaluation of the mutations (43, 44). Case 8 was identified as part of large-scale resequencing study of candidate ID genes using molecular inversion probes (MIPs). PPP2R5D was one of 42 candidate ID genes tested in 1,300 cases with a clinical diagnosis of ID and in whom previous molecular diagnostic tests were negative.

The 7 United Kingdom patients (cases 3–5, 10, and 12–14 in Tables 2 and 3) were recruited to the DDD study by the United Kingdom National Health Service or the Republic of Ireland Regional Genetics Service (3). Recruitment criteria were patients with neurodevelopmental disorders and/or congenital anomalies, abnormal growth parameters, dysmorphic features, and unusual behavior. DNA samples from patients and parents were analyzed by the Wellcome Trust Sanger Institute using high-resolution microarray analysis (array-CGH and SNP-genotyping) to investigate copy number variations (CNVs) in the child, and exome sequencing to investigate single nucleotide polymorphisms (SNPs) and small insertions/deletions (indels). Putative de novo variants were validated using targeted Sanger sequencing of blood-sample DNA. All genomic variants were annotated with the most severe consequence predicted by Ensembl Variant Effect Predictor (VEP) (45) and their minor allele frequencies observed in diverse population samples. Likely, diagnostic variants were fed back to referring clinical geneticists for validation and discussion with the family via the patient’s record in Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources (DECIPHER; Ensembl) (46), where they can be viewed in an interactive genome browser. Full genomic datasets were also deposited under accession number EGAS00001000775 in the European Genome-Phenome Archive (www.ebi.ac.uk/ega).

Biochemical investigations. To study the functional consequences of the de novo missense mutations, WT Aα and B56δ (isoform 1) cDNAs were cloned into HA-tag (pMB001) and EGFP-tag (pEGFP-P-C1) eukaryotic expression vectors, respectively. The different PPI2A B-subunit cDNAs were in a GST-tag eukaryotic expression vector, as described (19). PCR-based site-directed mutagenesis (Stratagene) was performed directly in the pMB001 or pEGFP vectors with proof-
The common feature in all ID cases described here is hindered access of phosphorylation by a competition-based, dominant-negative mechanism. The incorporation of a catalytically impaired C subunit into the trimeric complex result in protection of B subunit–directed substrate dephosphorylation. The pathological situation in which (i) a B subunit mutation hindering interaction of the A and C subunit, (ii) an A subunit mutation hindering interaction of the C subunit but not the B subunit, or (iii) an A subunit mutation resulting in the incorporation of a catalytically impaired C subunit into the trimeric complex result in protection of B subunit–directed substrate dephosphorylation by a competition-based, dominant-negative mechanism. The complex result in protection of B subunit–directed substrate dephosphorylation.

Figure 6. Mechanistic model. Top panels model the physiological situation in which the B-type subunit dictates subcellular targeting, subunit specificity, and substrate dephosphorylation by the C subunit. Conceivably, certain B subunits (like B56δ) or A-B dimers could dock to substrates independent of holoenzymes (alternatives labeled with question marks). Bottom panel displays the pathological situation in which (i) a B subunit mutation hindering interaction of the A and C subunit, (ii) an A subunit mutation hindering interaction of the C subunit but not the B subunit, or (iii) an A subunit mutation resulting in the incorporation of a catalytically impaired C subunit into the trimeric complex result in protection of B subunit–directed substrate dephosphorylation by a competition-based, dominant-negative mechanism. The common feature in all ID cases described here is hindered access of the PP2A activity to B56δ–specific PP2A substrates. S, substrate; P, phosphate.
these absolute values for amount of C present in the samples, as determined by immunoblotting with anti-C antibodies and quantification of the signals by ImageJ software.

For protein-stability analysis, HEK293 cells were transfected with EGFP-B56 (WT); with EGFP-B56-P53S or EGFP-B56-E198K mutants (pEGFP-C1); or with HA-As (WT), HA-As-R182W, or HA-As-P179L mutants (pMB001), one 10 cm plate per plasmid. Twenty-four hours after transfection, each 10-cm plate was split over 6 wells on a 6-well plate, in which eventually 50 μM cycloheximide (CHX, Sigma-Aldrich) was added per well to block translation. Following incubation with CHX for different time points (0, 10, and 24 hours), whole-cell lysates were prepared in NET lysis buffer and further analyzed by immunoblotting with anti-vinculin mouse monoclonals (Sigma-Aldrich), anti-HA, or anti-GFP antibodies. Band intensities were quantified using ImageJ software.

Statistics. Statistical analysis of biochemical data was done with 1-way multiple-comparisons ANOVA, and \( P < 0.05 \) was considered to be significant.

The calculation of the chance likelihood for finding 10 de novo mutations in the same 9–amino acid stretch of B56 was based on the following assumptions: The target size is \(<10^{-6} \) of the total ORF size, the number of random missense changes per generation is on average about 2,000 ID cases were tested. In that case, the phenotype should also be random, and this was not the case.

Study approval. The DDD study has UK Research Ethics Committee (REC) approval (10/H0305/83, granted by the Cambridge South REC, and GEN/284/12 granted by the Republic of Ireland REC). In other cases, ascertainment of patients was part of the clinical routine. All patients’ families have consented to publication of clinical findings. Written informed consent was also obtained for publication of all facial photographs presented in Figure 1.

Acknowledgments

The DDD study presents independent research commissioned by the Health Innovation Challenge Fund (HICF-1009–003), a parallel funding partnership between the Wellcome Trust and the Department of Health, and the Wellcome Trust Sanger Institute (WT098051). For details on the members of the study, see Supplemental Table 3. The views expressed in this publication are those of the author(s) and not necessarily those of the Wellcome Trust or the Department of Health. The research team acknowledges the support of the NIH Research, through the Comprehensive Clinical Research Network. Concerning the biochemical studies, we thank S. Dilworth for the gift of monoclonal PP2A-A subunit antibody and E. Heroes for the TEV-EGFP eukaryotic expression vector. Funding was provided by the KE Leven Research Fund (OT/13/094 to V. Janssens), the Research Foundation Flanders (G.0582.11 to V. Janssens), and the IAP program of the Belgian federal government (P7/13 to V. Janssens). D. Haesen received a fellowship of the Flemish Agency for Innovation by Science and Technology (IWT). A. Hoischen was supported by the Netherlands Organization for Health Research and Development (ZonMW 916-12-095). G. Houge was supported by HelseVest grant 91744.

Address correspondence to: Gunnar Houge, Center for Medical Genetics and Molecular Medicine, Haukeland University Hospital, N-5021 Bergen, Norway. Phone: 47.55.97.54.44; E-mail: gunnar.houge@helse-bergen.no. Or to: Veerle Janssens, Laboratory of Protein Phosphorylation and Proteomics, Gastroenterology and Genetic Medicine, Haukeland University Hospital, N-5021 Bergen, Norway. Phone: 47.55.97.54.44; E-mail: veerle.janssens@med.kuleuven.be.

20. Martens E, et al. Genomic organisation, chromo-
somal localisation tissue distribution and development


27. Dodge-Kafka KL, et al. cAMP-stimulated protein phosphatase 2A activity associated with muscle A kinase-anchoring protein (mAKAP) signaling complexes inhibits the phospho-


32. Dovega R, Tsutakawa S, Quistgaard EM, Ananda


42. Janssens V, Rebollo A. The role and therapeutic potential of Ser/Thr phosphatase PP2A in apop-


