De novo CCND2 mutations leading to stabilization of cyclin D2 cause megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndrome

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Activating mutations in genes encoding phosphatidylinositol 3-kinase (PI3K)-AKT pathway components cause megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndrome (MPPH, OMIM 603387)1–3. Here we report that individuals with MPPH lacking upstream PI3K-AKT pathway mutations carry de novo mutations in CCND2 (encoding cyclin D2) that are clustered around a residue that can be phosphorylated by glycogen synthase kinase 3β (GSK-3β)4. Mutant CCND2 was resistant to proteasomal degradation in vitro compared to wild-type CCND2. The PI3K-AKT pathway modulates GSK-3β activity5, and cells from individuals with PIK3CA, PIK3R2 or AKT3 mutations showed similar CCND2 accumulation. CCND2 was expressed at higher levels in brains of mouse embryos expressing activated AKT3. In utero electroporation of mutant CCND2 into embryonic mouse brains produced more proliferating transplanted progenitors and a smaller fraction of progenitors exiting the cell cycle compared to cells electroporated with wild-type CCND2. These observations suggest that cyclin D2 stabilization, caused by CCND2 mutation or PI3K-AKT activation, is a unifying mechanism in PI3K-AKT–related megalencephaly syndromes.

Germline mutations of PIK3R2 and AKT3 result in MPPH3, whereas postzygotic mutations of PIK3CA cause megalencephaly-capillary malformation syndrome4, and postzygotic mutations of PIK3CA and AKT3 cause isolated hemimegalencephaly1,5. We previously failed to detect mutations in PIK3R2 or AKT3 in 35% of probands with MPPH4. We hypothesized that mutation-negative patients have de novo mutations in one or more previously unreported genes, and thus we performed whole-exome sequencing in two child-parent trios (JT-144 and LR11-424) and one child-parent pair (LR02-064).

We identified germline mutations in CCND2 in all three affected individuals (resulting in p Thr280Ala in one subject and p.Lys270* in two unrelated subjects), and we confirmed these mutations as de novo by Sanger sequencing. None of the identified variants was present in dbsNP build 132, the 1000 Genomes Project, the National Heart, Lung, and Blood Institute (NHLBI) exome variant server or 6,500 exomes or in-house exome databases (Online Methods). We identified de novo heterozygous CCND2 mutations in nine additional patients with MPPH by conventional Sanger sequencing.

The clinical and molecular findings of this cohort are shown in Figure 1 and are summarized in Table 1, Supplementary Table 1 and Supplementary Note.

Received 12 July 2013; accepted 12 March 2014; published online 6 April 2014; doi:10.1038/ng.2948
All identified mutations affect highly conserved amino acid residues (Fig. 2 and Supplementary Fig. 1). Notably, 9 of 12 alterations involved Thr280 or Pro281 (Table 1). The p.Thr280Asn alteration occurred in three subjects, whereas p.Lys270*, p.Thr280Ala, p.Val284Gly or p.Pro281Arg were present in two subjects each. Mouse cyclin D2 can be phosphorylated at Thr280 in vitro by GSK-3β, rendering cyclin D2 susceptible to ubiquitin–proteasome-mediated degradation. The p.Thr280Ala cyclin D2 mutant cannot be phosphorylated and does not become polyubiquitinated and is thus resistant to degradation. Notably, all mutations in our MPPH cohort either alter highly conserved residues surrounding Thr280 or truncate the protein before this phosphorylation site (p.Lys270*). As the latter mutation occurs in the final exon, it is predicted to not induce nonsense-mediated mRNA decay.

To test whether mutations affecting particular residues in individuals with MPPH result in accumulation of degradation-resistant cyclin D2, we transfected HEK293 cells with either wild-type or mutant (encoding p.Thr280Ala, p.Pro281Arg, p.Val284Gly or p.Lys270*) human CCND2 Myc-tagged constructs. We treated cells with cycloheximide at 0.05 mg ml−1 at 24 h after transfection to block protein translation and analyzed protein extracts by western blotting (Fig. 3a,b). We observed a rapid reduction of cyclin D2 in cells transfected with wild-type constructs after cycloheximide treatment. We did not observe this effect in cells transfected with mutant constructs. Thus, MPPH–associated mutations in CCND2 result in accumulation of unphosphorylated, degradation-resistant cyclin D2.

Activation of the PI3K–AKT pathway inhibits GSK-3β–mediated phosphorylation. We hypothesized that the activating mutations previously observed in PIK3CA, PIK3R2 and AKT3 would also result in accumulation of degradation-resistant cyclin D2 through downstream inhibition of GSK-3β. We examined endogenous CCND2 levels in lymphoblastoid cell lines derived from patients with mutations in PIK3CA (resulting in p.Glu453del), PIK3R2 (resulting in p.Gly373Arg) or AKT3 (resulting in p.Arg465Trp). After cycloheximide treatment, we observed a rapid decrease in CCND2 levels in control cells. In contrast, the CCND2 in cells from patients was resistant to degradation (Fig. 3c,d). These results suggest that megalencephaly-associated mutations altering components of the PI3K–AKT pathway share the same functional endpoint as the mutations observed in CCND2—namely, inhibition of proteasomal degradation of cyclin D2.

The PI3K–AKT pathway inactivates GSK-3β by phosphorylation of Ser9 (ref. 6), which should interfere with GSK-3β–dependent phosphorylation, ubiquitination and proteasomal degradation of cyclin D2. Activating mutations previously described in PIK3R2 and PIK3CA result in AKT3 activation3. To further test the relationship between cyclin D2 and this pathway in vivo, we examined the levels of CCND2 in embryonic day (E) 14.5 cortices of mice homozygous for an allele of Akt3 carrying the gain–of-function mutation encoding p.Asp219Val; this is referred to as Akt3D219V in the original publication5. Quantitative western blot analysis showed a 30% increase in the levels of phosphorylated Ser9 (pSer9), corresponding to the inactive GSK-3β phosphoprotein, associated with a 30–40% increase in cyclin D2, which was equivalent to the increase in cyclin D1 (Fig. 3e).

Neocortical neurons and glia are generated in the embryonic ventricular and subventricular zones (VZ and SVZ, respectively)6. Cyclin D2 promotes neuronal progenitor commitment to undergo a round of division and progress through the cell cycle8–11. In the embryonic mouse brain, CCND2 is expressed in some VZ radial glial cells (RGCs) that colabel for Pax6, but CCND2-positive cells colocalize primarily (though certainly not exclusively) with Tbr2-positive intermediate progenitor cells (IPCs) in the SVZ12,13. IPCs are important drivers of brain size, as they allow for geometric expansion of cellular output from the SVZ10. Ccnd2-null mice display microcephaly and cerebellar hypoplasia14,15. In the human neocortex, cyclin D2 is expressed more robustly than cyclin D1 by 19 gestational weeks15. Thus, elevated CCND2 levels may enlarge brain size by maintaining cells in cycle and by increasing progenitor numbers, whereas diminished CCND2 expression reduces brain volume by allowing cell cycle exit, resulting in depletion of the progenitor pool14.
including those found in individuals with MPPH, using in utero electroporation (IUEP) in mouse embryos (Online Methods). Western blotting of HEK293 cells transfected with these constructs demonstrated that CCND2 sequences placed upstream of the internal ribosome entry site–enhanced GFP (IRES-eGFP) cassette were translated into protein (Supplementary Fig. 2). Immunostaining of the embryonic cortex after IUEP confirmed that expression of nuclear CCND2 in GFP-positive cells was elevated after transfection with mutant forms of CCND2 compared to cells transfected with GFP only or with untransfected cells (i.e., GFP-negative cells) (Supplementary Fig. 2).

**Table 1** Summary of phenotypic and molecular data for 12 individuals with MPPH harboring CCND2 mutations

| Subject ID | Sex | Age last assessed | First OFC s.d. (age) | Last OFC s.d. (age) | HYD or VMEG | Shunt, age at shunting | PMG | Polydactyly | ID | cDNA change | Amino acid change | Inheritance |
|-----------|-----|-------------------|---------------------|---------------------|-------------|----------------------|-----|-------------|----|-------------|-------------------|-------------|
| LR02-064 | F   | 6 y               | +3 (5 d)            | +3 (6 y)            | HYD         | +, 6 y               | BPP grade 1 | ++/++       | ID | c.808A>T    | p.Lys270*         | De novo     |
| LR11-424 | F   | 18 m              | +4 (birth)          | +4 (18 m)           | VMEG        | –                    | BPP grade 2 | +/+         | ID | c.808A>T    | p.Lys270*         | De novo     |
| JT-144.1 | M   | 3 y               | +3 (birth)          | +2.5 (3 y)          | VMEG        | –                    | BPP grade 2 | +/+         | ID | c.838A>G    | p.Thr280Ala      | De novo     |
| VI13216  | M   | 7 y               | +4 (birth)          | +7.5 (7 y)          | VMEG        | –                    | BPP grade 1 | ++/−−       | ID | c.838A>G    | p.Thr280Ala      | De novo     |
| LR07-041 | F   | 8 m               | +4 (6 w)            | +2 (8 m)            | VMEG (mild) | –                    | BPP grade 1 | ++/−−       | ID | c.839C>A    | p.Thr280Asn      | De novo     |
| LR11-352 | F   | 13 y              | +4.5 (2 wk)         | +5 (13 y)           | VMEG        | –                    | BPP grade 2 | ++/++       | MILD LD, ambulatory, ID | c.839C>A    | p.Thr280Asn      | De novo     |
| JT-232   | M   | 5 y               | +3.5 (2 d)          | +6 (5 y)            | VMEG        | –                    | BPP grade 2 | −−/++       | ID | c.839C>A    | p.Thr280Asn      | Parents NA   |
| LR11-346 | F   | 28 wk             | MEG                 | NA                  | VMEG (mild) | –                    | Frontoparietal PMG | +++/−− | ID | c.841C>T    | p.Pro281Ser      | De novo     |
| LP95-025 | M   | 20 y              | +3 (y)              | +2.5 (8 y)          | HYD         | +, 1 m               | BPP grade 2 | −−/++       | ID | c.842C>G    | p.Pro281Arg      | De novo     |
| JT-210   | F   | 2 y               | +2 (birth)          | +2 (2 y)            | VMEG (mild) | –                    | BPP grade 2 | ++/−−       | ID | c.842C>G    | p.Pro281Arg      | De novo     |
| LR03-260 | M   | 9 y               | +2 (birth)          | +5 (9 y)            | VMEG        | –                    | BPP grade 1 | ++/++       | ID | c.842C>G    | p.Pro281Arg      | De novo     |
| JT-238   | F   | 11 y              | +2.5 (birth)        | +3.5 (11 y)         | VMEG (L>R)  | –                    | BPP grade 1 | ++/++       | ID | c.851T>G    | p.Val284Gly      | Mother negative, father NA |

BPP, bilateral peri- and polymicrogyria; HYD, hydrocephalus; ID, intellectual disability; LD, learning disability; MEG, megalencephaly; NA, not available or not applicable; OFC, occipitofrontal circumference; PMG, polymicrogyria; VMEG, ventriculomegaly.

*The BPP grades are as follows: grade 1, PMG extending beyond the perisylvian regions to the frontal or occipital poles or both; and grade 2, PMG extending beyond the perisylvian regions but not to either pole. aPostaxial polydactyly indicated for both hands and both feet (hands/feet). bSubjects analyzed by exome sequencing. Mutation locations are based on reference gene accession NM_001759.3 (CCND2). All mutations were absent from the Exome Variant Server, which consists of whole-exome sequencing data from 6,500 subjects (accessed January 2012). cThis child’s non-identical twin sister is healthy. dThis child had progressively worsening irritability, severe episodes of opisthotonos, Cheyne-Stokes respirations and poor eye contact and died at 8 months of age. eThis pregnancy was terminated at 28 weeks of gestation. On autopsy examination, the brain weighed three times the normal weight. fThis child walked unassisted at 5 years. Her speech is limited to two-word phrases. gThis pregnancy was terminated at 28 weeks of gestation. On autopsy examination, the brain weighed three times the normal weight. hThis child has cortical visual blindness and a ventricular septal defect that closed spontaneously.

We performed IUEP at E13.5 and collected embryos 48 h later. Immunohistochemistry using antibodies to GFP and Ki67 (Fig. 4) showed that 85% of control cells expressing GFP alone did not colabel for Ki67 and therefore had exited the cell cycle; a large proportion of these cells had migrated to the cortical plate (Fig. 4a, f, k). Significantly more GFP-positive cells expressing wild-type CCND2 also colabeled for Ki67 and were actively dividing (27.6 ± 0.4% of control cells expressing only GFP) (Fig. 4b, g, k). Immunostaining of the embryonic cortex after IUEP confirmed that expression of nuclear CCND2 in GFP-positive cells was elevated after transfection with mutant forms of CCND2 compared to cells transfected with GFP only or with untransfected cells (i.e., GFP-negative cells) (Supplementary Fig. 2).
We investigated the effects of MPPH-associated CCND2 mutation on cell cycle function by determining the proportion of electroporated (GFP-positive) cells undergoing mitosis. We used immunodetection of phosphohistidine H3 (pH3), a marker of cells in M phase, after transfection with constructs encoding wild-type, phosphodefective (p.Thr280Ala) and phosphomimetic (p.Thr280Asp) forms of CCND2. The phosphodeficient CCND2 sequence resulted in a lower number of progenitors in the P fraction (45.4 ± 1.4%) and exit fraction (54.6 ± 1.4%) relative to transfection with wild-type CCND2 (Fig. 5e,f,h,i,k and Supplementary Table 3). We then investigated how transfection of cortical progenitors with wild-type and mutant forms of CCND2 would affect the RGC and IPC populations. Previous work has detailed the transition of progenitors from Pax6-expressing RGCs to Tbr2-positive IPCs during development within the cortex16,17. Therefore, we examined the expression of Pax6 and Tbr2 in electroporated progenitors. First we examined the proportion of electroporated progenitors that coexpress Pax6. Introduction of the control construct expressing GFP only generated...
Figure 4  Comparative ability of mutant and wild-type CCND2 to enhance neural progenitor proliferation in the embryonic mouse brain. Two patient mutations (encoding p.Thr280Ala and p.Pro281Arg) are compared to wild-type and phosphomimetic (p.Thr280Asp) CCND2. Wild-type and mutant cDNAs were cloned into an IRES-eGFP vector, affording independent translation of both CCND2 and, from an IRES, eGFP. Electroporated (GFP+ cells expressed either GFP alone or GFP together with human wild-type or mutant CCND2. Constructs (1 µg each) were delivered to the cortex by IUEP at E13.5, and embryos were harvested at E15.5. (a–e) Cortical sections were immunolabeled with antibodies to GFP (green) and Ki67 (red). Ki67 is a proliferation marker. (a,f) For the most part, cells receiving vector without CCND2 are no longer dividing, and many have migrated up to the cortical plate. (b,g) More cells continue to divide (yellow cells, mostly in the SVZ and intermediate zone (IZ)) when overexpressing wild-type CCND2. (c,h,e,j) Progenitors expressing either the phosphodeficient p.Thr280Ala CCND2 or putative phosphodeficient p.Pro281Arg CCND2 found in patients with MPPH show strikingly more proliferation and fewer GFP+ cells residing in the cortical plate. (d,i) A phosphomimetic (p.Thr280Asp) form of CCND2 is significantly less effective in promoting proliferation than the forms carrying MPPH-associated mutations. (k) Quantification of the proportions of transfected (GFP+) cells that are proliferating (GFP+Ki67+ cells) or nonproliferating (GFP+Ki67− cells). Error bars, s.e.m. *P < 0.05, **P < 0.0001 with respect to cells expressing GFP only (control); §P < 0.0001 with respect to cells expressing wild-type or p.Thr280Asp CCND2, as indicated. A full statistical summary is shown in Supplementary Table 2, and the P values shown in the figure were calculated by one-way analysis of variance (ANOVA) with post-hoc tests (LSD). n = 7 embryos (a), n = 5 embryos each (b–e), with each value an average of five sections per embryo. MZ, marginal zone; CP, cortical plate. Scale bar, 20 µm.

21.5 ± 1.5% of GFP-positive cells that were positive for Pax6 as well (Supplementary Fig. 3a). Electroporation with either wild-type CCND2 or the phosphomimetic CCND2 construct did not result in an increase in the proportion of progenitors that were immunopositive for Pax6 (20.1 ± 1.1% and 23.0 ± 0.6%, respectively). In contrast, electroporation of the phosphodeficient CCND2 construct caused an

Figure 5  Phosphodeficient CCND2 more effectively promotes proliferation and prevents neural progenitor cells from exiting the cell cycle. (a–d) Tissue sections immunostained with antibodies to GFP (green) and pH3 (purple) after electroporation with constructs encoding wild-type, phosphodeficient (p.Thr280Ala) and phosphomimetic (p.Thr280Asp) CCND2. (a–c) Compared to transfection with wild-type CCND2, introduction of phosphodeficient CCND2 resulted in a significant increase in the number of GFP+ and pH3+ double-labeled cells in the VZ and SVZ (a,b), whereas transfection with phosphomimetic CCND2 was no different from transfection with wild-type CCND2 (c). (d) Quantification shows significantly more GFP+Pax6+ cells (e–g) were delivered to the cortex by IUEP at E14.5 (24 h after IUEP) and harvested at E15.5. (h–j) The same samples were also co-labeled with antibodies to Ki67 (blue). Expression of either the wild-type form of CCND2 (e,h,k) or phosphomimetic (p.Thr280Asp) forms of CCND2 (g,j,k) resulted in a similar increase in the percentage of progenitors in the exit fraction over those in the P fraction (Online Methods), thereby indicating that more cells had exited the cell cycle 24 h after BrdU injection. (f,i,k) Introduction of the phosphodeficient (p.Thr280Ala) form of CCND2 significantly increased the number of progenitors in the P fraction (still proliferating 24 h after BrdU pulse) and correspondingly decreased the number in the exit fraction compared to transfection with either the wild-type or phosphomimetic form of CCND2. Thus, phosphodeficient CCND2 was more effective in promoting mitotic events and keeping cells in cycle. Full statistical analyses are shown in Supplementary Table 3. GFP and pH3 experiment: n = 4 embryos, wild-type CCND2; n = 5 embryos, p.Thr280Ala; n = 5 embryos, p.Thr280Asp. GFP; BrdU and Ki67 experiment: n = 5 embryos, wild-type CCND2; n = 5 embryos, p.Thr280Ala; n = 5 embryos, p.Thr280Asp. Each value shown is the mean ± s.e.m. Scale bars, 20 µm. *P < 0.02 compared to wild-type CCND2, **P < 0.01 compared to p.Thr280Ala CCND2. Throughout the figure, unpaired Student’s t-test was used to calculate all P values.
increase in the proportion of GFP-positive progenitors that coexpress Pax6 (27.7 ± 1.4%) over GFP-only control (P = 0.003), wild-type (P < 0.0001) and phosphomimetic (P = 0.023) constructs, indicating that phosphodeficient CCND2 leads to expansion of RGCs in the developing cortex. We then found that introduction of the construct expressing GFP only resulted in 9.4 ± 0.7% of GFP-positive cells also expressing Tbr2 (Supplementary Fig. 3b). In contrast, expression of wild-type CCND2 or the phosphomimetic form generated significant increases in the proportion of electroporated cells (17.6 ± 2.1%, (P = 0.006) and 15.3 ± 1.7% (P = 0.046), respectively) that were also immunopositive for Tbr2, signifying an expansion of the IPC population. The phosphodeficient CCND2 mutant produced an even greater (27.4 ± 2.9%) expansion of progenitors expressing both GFP and Tbr2, which was markedly different from the control group expressing only GFP (P < 0.0001) and the groups expressing wild-type CCND2 (P = 0.002) or phosphomimetic CCND2 (P = 0.001). Thus, the phosphodeficient, degradation-resistant form of CCND2 causes an expansion of both the RGC and IPC populations. We suggest that it is this expansion that underlies the megalencephaly in individuals with MPPH. Wild-type CCND2 resulted in an increase in the proportion of transfected progenitors that coexpress Tbr2 but not Pax6, suggesting that wild-type CCND2 might preferentially promote expansion of the IPC population.

Polymicrogyria in individuals with MPPH may be related to an altered relationship between basal progenitors and radial glia. A homozygous loss-of-function mutation in EOMES (also called TBR2), expressed in IPCs, was previously associated with a microcephaly-polygyria relationship between basal progenitors and radial glia. A homozygous expansion of the IPC population.

Thus, the phosphodeficient, degradation-resistant form of CCND2 causes an expansion of both the RGC and IPC populations. We suggest that it is this expansion that underlies the megalencephaly in individuals with MPPH. Wild-type CCND2 resulted in an increase in the proportion of transfected progenitors that coexpress Tbr2 but not Pax6, suggesting that wild-type CCND2 might preferentially promote expansion of the IPC population.

Polymicrogyria in individuals with MPPH may be related to an altered relationship between basal progenitors and radial glia. A homzygous loss-of-function mutation in EOMES (also called TBR2), expressed in IPCs, was previously associated with a microcephaly-polygyria phenotype18. In the fetal brain, CCND2 is expressed mostly in IPCs, and altered cyclin D2 levels are expected to disrupt the proportion of IPC-compared to RGC-derived neurons and/or the differentiation program of IPCs14. We note that cases with MPPH carrying CCND2 mutation have polydactyly more frequently than those with P13K-AKT pathway mutations (Supplementary Table 1), but the mechanism underlying this phenotypic difference remains to be explored.

Thus, we have identified a highly specific pattern of de novo mutations in CCND2 in 12 subjects with MPPH. We and others previously reported activating mutations in P13K-AKT pathway elements upstream of CCND2 in other subjects with MPPH and overlapping megalencephaly syndromes1–3. Our evidence presented here supports that these two types of mutational events result in a common functional endpoint—namely, increased cyclin D2 activity in neural precursors as a result of its intracellular accumulation. The data suggest that pathological stabilization of cyclin D2 in the developing cerebral cortex may be a unifying mechanism for the development of megalencephaly and polymicrogyria in MPPH and other P13K-AKT-associated megalencephaly syndromes (Supplementary Fig. 4).

METHODS

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

URLS. FastX-Toolkit, http://hannonlab.cshl.edu/fastx_toolkit/; GATK, http://www.broadinstitute.org/gsa/wiki;/Picard tools, http://picard.sourceforge.net/; SAMTools, http://samtools.sourceforge.net/; HomoloGene, http://www.ncbi.nlm.nih.gov/homologene.

ACKNOWLEDGMENTS

We thank the study patients and their families, without whose participation this work would not be possible. We thank M. O’Driscoll (University of Sussex) for advice and help. This work was funded by the Government of Canada through Genome Canada, the Canadian Institutes of Health Research (CIHR), the Ontario Genomics Institute (OGI-049), Genome Quebec and Genome British Columbia (to K.M.B.). The work was selected for study by the FORGE Canada Steering Committee, consisting of K. Bear, University of Ottawa, J. Friedman (University of British Columbia), J. Michaud (University de Montreal), F. Bernier (University of Calgary), M. Brudno (University of Toronto), B. Fernandez (Memorial University), B. Knoppers (McGill University), M. Samuels (Université de Montreal) and S. Scherer (University of Toronto). Research reported in this publication was supported by the National Institute of Neurological Disorders and Stroke (NINDS) of the US National Institutes of Health under award numbers P01-NS048120 (to M.E.R.), NRSA F32 NS086173 (to K.A.G.) and 1R01NS058721 (to W.B.D.), by The Baily Thomas Charitable Fund (to D.T.P) and by the Sir Jules Thorn Charitable Trust and Great Ormond Street Children’s Hospital (to E.G.S.).

AUTHOR CONTRIBUTIONS

E.G.S., W.B.D., D.T.P., M.E.R., K.M.B., G.M.M., D.A.P., A.E.F. and K.A.G. designed the study and experiments. G.M.M., A.E.F., C.A., D.T.B., K.P.I.B., L.F., K.W.G., G.M.S.M., K.P., E.S., H.v.E., N.V., D.W., D.T.P., W.B.D. and E.G.S. identified, consented and recruited the study subjects and provided clinical information. J.-B.R., J.S.-O. and M.S. recruited patients. G.M.M., A.E.F., D.T.P. and W.B.D. evaluated the magnetic resonance imaging. G.M.M., D.A.P., C.A.J., S.M., C.V.L. and N.R. developed the bioinformatics scripts and performed genetic data analysis and expression studies. R.D.H. and R.P. provided the AKT3 mouse mutant brain samples. D.A.P. and C.V.L. performed the protein stability experiments. K.A.G., S.S., S.S.K. and M.E.R. performed and analyzed the IUEP experiments and quantitative western blot analyses of p.Asp219Val AKT3. J.M. analyzed data. G.M.M., D.A.P., A.E.F., K.A.G., D.T.P., M.E.R., W.B.D. and E.G.S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Whole-exome sequencing. Alignment and variant calling (JT144). Target capture was performed using the Agilent SureSelect All Exon v4 exome enrichment kit according to standard manufacturer protocols. Sequencing of 150-bp paired end reads was performed using an Illumina MiSeq. Reads were aligned to GRCh37 using Novoalign (Novocraft Technologies) and processed using the Genome Analysis Toolkit (GATK) and Picard (see URLs) to perform realignment of short insertions and deletions (indels) and remove duplicate reads. The depth of coverage of the consensus coding sequence (CCDS) was assessed using the GATK, which showed that >94% of CCDS bases were covered by at least five good-quality reads (minimum Phred-like base quality 17 and minimum mapping quality 20). Single-nucleotide variants (SNVs) and indel variants were called using the UnifiedGenotyper feature of the GATK.19,20

Filtering (JT144). Custom Perl scripts were used to remove variants present in dbSNP132 or with a minor allele frequency ≥0.1% and to annotate functional consequences. We identified variants present in a child but not in her or her parents with a minimum Phred-like genotype quality of 30 and selected for indels within coding regions, nonsynonymous SNVs and splice-site variants. Variants present in the 1000 Genomes data set (November 2011), the NHLBI GO exomes and another 22 ethnically matched in-house exomes were also removed. This left four de novo variants (CCND2 (NM_001759) c.383A>G, p Thr280Ala; HVNL (NM_144570) c.98C>G, p.Pro20Ala; GGG3 (NM_012201) c.1229A>G, p.His410Arg; and RFG3 (NM_011309) c.387del, p.Ala131Argfs*16), which were confirmed to be present in the child and absent in the parents by Sanger sequencing.

Alignment and variant calling (LR11-424 and LR02-064). Target capture was performed using the Agilent SureSelect All Exon 50 Mb enrichment kit according to standard manufacturer protocols. Sequencing of 100-bp paired end reads was performed using an Illumina HiSeq 2000, which generated over 13 Gb of sequence for each sample. We removed adaptor sequences and quality trimmed reads using the Fastskit tool (see URLs) and then used a custom script to ensure that only read pairs with both mates present were subsequently used. Reads were aligned to hg19 with BWA 0.5.9 (ref. 21), and indel realignment was done using GATK.20 Duplicate reads were marked using Picard (see URLs) and excluded from downstream analyses. We assessed coverage of CCDS bases using the GATK, which showed that all samples had ≥92.7% of CCDS bases covered by at least 10 reads and ≥88.4% of CCDS bases covered by at least 20 reads. SNVs and indels were called using SAMtools mpileup with the extended base alignment quality (BAQ) adjustment (–E) and were then quality filtered to require at least 20% of reads supporting the variant call.22 Variants were annotated using both Annovar23 and custom scripts to annotate functional consequences and whether they had been seen previously in dbSNP132, the 1000 Genomes data set (November 2011), the NHLBI GO exomes or in approximately 570 exomes previously sequenced at our centers.

Filtering (LR11-424 and LR02-064). We removed all variants seen in the parents of the affected subjects or in any of our 570 controls samples, the 6,500 NHLBI GO exomes or the 1000 Genomes Project. We focused on nonsynonymous SNVs, indels and splice-site variants. This analysis revealed a single de novo variant in CCND2 (encoding p.Lys270*) in patient LR11-424. Surprisingly, LR02-064 had the same CCND2 variant (encoding p.Lys270*), which was not present in the single parent that was exome sequenced.

Constructs for site-directed mutagenesis and transfection. A C-terminal Myc-tagged cDNA clone of CCND2 transcript NM_001759 was purchased from Origene (RC210536). The constructs with the mutations encoding p Thr280Ala, p.Pro281Arg or p.Val284Gly were created using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) as per the manufacturer’s instructions with the primer pairs shown in Supplementary Table 4.

Transfection and protein extractions. HEK293 cells were transfected with wild-type or mutant CCND2 constructs using Lipofectamine 2000 Transfection Reagent (Life Technologies) as per the manufacturer’s instructions. The medium was changed 24 h after transfection for medium containing 50 µg/ml cycloheximide (Sigma-Aldrich) to block protein translation. Protein was extracted at 0-, 60-, 120- and 240-min time points using NP-40 lysis buffer (150 mM sodium chloride, 1.0% NP-40 and 50 mM Tris, pH 8.0) containing protease and phosphatase inhibitors (Thermo Scientific).

Lymphoblastoid cell line treatment and protein extraction. Lymphoblastoid cell lines derived from patients with mutations in PIK3R2 (encoding p.Gly373Arg), PIK3CA (encoding p.Glu542del) or AKT3 (encoding p.Arg465Trp) were treated with 25 µg/ml cycloheximide (Sigma-Aldrich) for 0, 30, 60 or 90 min, and protein was extracted as described above.

Western blotting of the CCND2-Myc-transfected cell line and patient lymphoblastoid protein extracts. Equal amounts of protein were heated to 70 °C for 10 min with NuPAGE LDS Sample Buffer (4×) (Life Technologies), run on NuPAGE Novex 4–12% Bis-Tris gels (Life Technologies) and transferred to polyvinylidene difluoride (PVDF) membranes (Life Technologies). Membranes were blocked for 1 h at room temperature with 5% dried skimmed milk (Marvel) in PBS with 0.1% Tween-20. Membranes were then incubated with primary antibody at a dilution of 1:200 for CCND2 (ab3085, Abcam) and 1:10,000 for β-actin (ab8226, Abcam) overnight in blocking solution at 4 °C with slight agitation. The membranes were washed with 0.1% Tween-20 in PBS and then incubated with secondary antibody for 1 h at room temperature. The secondary antibody (polyclonal goat anti-mouse immunoglobulins/ horseradish peroxidase (HRP), P0447, Dako) was diluted 1:10,000 in blocking solution. Membranes were then washed in PBS with 0.1% Tween-20, developed with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific) and visualized using a Gelsdoc XR system (Bio-Rad). Experiments were performed in triplicate, and tiff images were analyzed using ImageJ.24 Bands were quantified and intensities were determined relative to the corresponding 0 time point. CCND2 intensity was normalized with β-actin.

Generation of constructs for in utero electroporation. The ORF of human CCND2 was cloned into the pcAGIG vector (iRES-eGFP, Addgene) at the multiple cloning site after digestion with EcoRI and XhoI. Mutations encoding phosphodeficient (p Thr280Ala), phosphomimetic (p Thr280Asp) and patient (p.Pro281Arg) alterations were made using the QuikChange Site-Directed Mutagenesis Kit (Supplementary Table 4) at the manufacturer’s instructions. The p Thr280Ala phosphodeficient alteration involved the following change: ACC (threonine) to GCC (alanine); the phosphomimetic mutation entailed altering the first mutant: GCC (alanine) to GAC (aspartic acid); and the p.Pro281Arg alteration changed CCT (proline) to CGT (arginine). Constructs were then sequenced to confirm the mutations. There were five experimental groups: GFP control, wild-type CCND2 and the p.Pro281Arg alteration and two phosphodeficient (p Thr280Ala and p.Pro281Arg) alterations were made using the QuikChange Site-Directed Mutagenesis Kit (Supplementary Table 4). All experiments were performed in accordance with protocols approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee.

Animals. Timed pregnant female wild-type CD-1 mice at least 10 weeks of age were obtained from Charles River Laboratories and then maintained as part of our laboratory colony and were housed at 23 °C with a 12 h light, 12 h dark cycle in Thoren units. The day of plug discovery (12 p.m.) was designated as E0.5. All experiments were performed in accordance with protocols approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee.

In utero electroporation and BrdU injections. Electroporations were carried out at E13.5. The pregnant dam was anesthetized with isoflurane (Butler Schein), and a laparotomy was performed to expose the uterus. DNA was diluted 1:20 in Fast Green (Sigma-Aldrich) to achieve a final concentration of 1 µg/µl. Then, 1 µg DNA was injected into the lateral ventricle of each embryo. Tween20 (7 mm platinum, BTX, Harvard Apparatus) were placed over the injection site, and then five 50-ms pulses at 42 V were delivered with 1-s intervals between each pulse using a BTX-ECM830 electroporator (BTX, Harvard Apparatus) to introduce DNA into the cortex. Embryos were returned to the abdominal cavity, and the wound was sutured; dams were allowed to recover and were monitored closely. Embryos were harvested at 48 h after IUEP at E15.5. Embryo brains were collected in cold 1× PBS and screened for GFP expression using a stereomicroscope (Leica M165 FC). Brains were transferred to 4% paraformaldehyde in 1× PBS with 7% sucrose overnight at 4 °C. The next day, brains were transferred to 15% sucrose and then to 30% sucrose in 1× PBS, shaking at 4 °C, until properly sunk. Brains were then embedded in optimal cutting temperature solution (Electron Microscopy Sciences) and stored at –20 °C.
A subset of pregnant dams were injected with BrdU (Sigma-Aldrich) at 50 mg/kg at E14.5 (24 h after IUEP). All tissue was then harvested at E15.5 (48 h after IUEP).

Immunohistochemistry. Brains were sectioned coronally at 12 µm on a cryostat (HM505E, Microm) and collected on glass slides. Sections to be stained were washed three times with 1× PBS with 0.3% Triton X-100 (PBS-T) and then subjected to antigen retrieval with steam in 1 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM Tris, pH 8 (GFP and Pax6 dual labeling), for 20 min or 10 mM sodium citrate buffer, pH 6, for 15 min (for all other staining). Slides were allowed to come to room temperature, were rinsed in 1× PBS-T and were then blocked for 1 h at room temperature in 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc.) in 1× PBS-T. Antibodies were diluted in blocking medium and were used as follows: anti-GFP (600-101-215, goat IgG, Rockland Immunocchemicals, Inc., 1:10,000), anti-Ki67 (RM-9106-S1, rabbit IgG, Thermo Scientific, 1:1,000), anti-CCND (60-570, rabbit IgG, Millipore, 1:2,000), anti-BrdU (555627, mouse IgG, BD Biosciences, 1:500), anti-PAX6 (PRB-278P, rabbit IgG, Covance, 1:1,000) and anti-TBR2 (ab23345, rabbit IgG, Abcam, 1:2,000). Primary antibodies were incubated overnight at 4 °C. The next day, slides were washed three times in 1× PBS-T and incubated with Alexa fluorescence-conjugated secondary antibodies (donkey anti-goat IgG, 488, A11055; donkey anti-rabbit IgG, 568, A10042; donkey anti-rabbit IgG, 647, A31573; donkey anti-mouse IgG, 686, A10037; Molecular Probes, Life Technologies, 1:1,000 in blocking solution) for 2 h at room temperature. Slides were then washed and coverslipped with ProLong Antifade Kit (Life Technologies).

An additional set of brains was processed for paraffin sectioning as previously described14. Sections were collected on 4 µm and first processed for anti-CCND2 immunohistochemistry (MS-221-P0, mouse IgG, Thermo Scientific, 1:100)14. After the primary antibody incubations, sections were incubated in biotinylated secondary antibody (biotinylated horse anti-mouse IgG, 647, A31573; donkey anti-mouse IgG, 686, A10037; Molecular Probes, Life Technologies, 1:1,000 in blocking solution) for 2 h at room temperature. Slides were then washed and coverslipped with ProLong AntiFade Kit (Life Technologies).

Western blotting of GFP constructs. Samples were reduced and boiled at 70 °C for 10 min. Equal amounts of protein were run on polyacrylamide gels as described above (Invitrogen) and then transferred to nitrocellulose membranes (Whatman). Membranes to be stained with antibodies to CCND2 and GFP were blocked in 5% nonfat dry milk in 1× TBS-T. Membranes were then incubated in primary antibodies as follows: anti-CCND2 (sc-53637/sc-56305, mouse IgG, Santa Cruz Biotechnology, Inc., 1:1,000) and anti-GFP (as described above, goat IgG, 1:4,000 dilution in block solution overnight at 4 °C with slight agitation. The next day, membranes were washed and then incubated in secondary antibody (1 h at room temperature diluted in Odyssey LI-COR solution with 4% baking solution) for 2 h at room temperature. Fluorescent secondary antibody incubations were carried out as described above.

Microscopy and cell quantifications. Sections were imaged with a 20× objective using an epifluorescent microscope (Leica DM550B with Leica Application Microscopy and cell quantifications. For all IUEP experiments, embryo brains were assigned to appropriate experimental groups. For the GFP and PAX6 and TBR2 experiments, at least 6,400 GFP+ cells per experimental group were counted (n = 6, GFP; n = 6, wild-type CCND2; n = 5, p.Thr280Ala; n = 5, p.Thr280Asp; 5 sections per embryo).

Final counts are presented as percentages ± standard error measurement. Unless otherwise noted, a two-tailed unpaired Student’s t-test was performed, with p < 0.05 considered significant. For Figure 4 and the Pax6 and Tbr2 experiments (Supplementary Fig. 3), one-way ANOVA was used, where p < 0.05 was deemed significant. Post hoc tests (LSD) were then performed to corroborate the independent t-tests. Pairwise comparisons were not adjusted for the multiple comparisons because all groups were defined a priori. In this case, the statistical values shown were from the post hoc tests after ANOVA analysis. Statistics were calculated using Microsoft Excel and SPSS (Version 22, IBM). All analyzed data met the assumptions of two-tailed unpaired Student’s t-test or one-way ANOVA, and the variance between experimental groups was similar. For all in utero experiments, we estimated we would need 4–7 embryos per group on the basis of studies previously performed in our laboratory.13 Graphs were made in Microsoft Excel, and final figures were compiled in Adobe Photoshop 12.0 (Adobe Systems).

Cell culture. HEK293 cells (from ATCC) were transfected with the IUEP constructs using XFect (Clontech Laboratories Inc.) according to the manufacturer’s instructions and then collected 48 h later. Cells were lysed using Mammalian Protein Extraction Reagent (Thermo Scientific) with protease and phosphatase inhibitors in addition to EDTA and then flash frozen until needed for western blotting.

Western blotting of GFP constructs. Samples were reduced and boiled at 70 °C for 10 min. Equal amounts of protein were run on polyacrylamide gels as described above (Invitrogen) and then transferred to nitrocellulose membranes (Whatman). Membranes to be stained with antibodies to CCND2 and GFP were blocked in 5% nonfat dry milk in 1× TBS-T. Membranes were then incubated in primary antibodies as follows: anti-CCND2 (sc-53637/56305, mouse IgG, Santa Cruz Biotechnology, Inc., 1:1,000) and anti-GFP (as described above, goat IgG, 1:4,000 dilution in block solution overnight at 4 °C with slight agitation. The next day, membranes were washed and then incubated in secondary antibody (1 h at room temperature diluted in Odyssey LI-COR solution with 0.05% Tween 20 using LI-COR secondary antibodies (1:10,000, donkey anti-mouse IRDye 800CW and donkey anti-goat IRDye 680LT). Blots were then visualized on the Odyssey FC imaging system (LI-COR Biosciences). Quantification was performed by serial dilution of lysates, and fluorescent values within the linear range were used for determining relative levels normalized to the loading control of total GSK-3β. Primary antibodies used in the quantitative western blot analyses included anti-CCND2 (as described above), anti-CCN1 (ab134175, rabbit IgG, Abcam, 1:4,000); anti-CCND2 (9832, mouse IgG, Cell Signaling, 1:2,000) and anti-GSK-3β (pSer9) (9292, rabbit IgG, Cell Signaling, 1:1,000).

Western blotting of AKT3 p.Asp219Val embryo brain lysates. Embryo brains were harvested at E14.5. Lysates were prepared, and equal amounts of protein were loaded onto polyacrylamide gels for western blot transfer and immunodetection, as described above for the cell cultures. A total of three wild-type and AKT3 p.Asp219Val and p.Asp219Val littermate pairs were analyzed, providing three biological replicates. Each wild-type and AKT3 p.Asp219Val p.Asp219Val pair was processed in parallel as follows: 21, 18, 15, 12, 9 or 6 µl of lysate was loaded on a gel. Out of six data points for each biological replicate, at least three were in the linear range, giving three technical replicates for each sample, for a total of n = 9. Wild-type and mutants were then compared using a sign test, and for every wild-type and mutant pair from the same litter and of the same dilution, the sign of the difference was the same (for example, CCND2 or CCND1 was greater in the mutant than the wild-type littermate), for a two-sided level of significance of 0.02.

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