Supplementary note:
Research Subjects

Informed consent was obtained from all subjects, and the study was approved by the institutional review boards at participating institutions: St James's University Teaching Hospital, Leeds UK, Institute of Medical Genetics, University Hospital of Wales, Cardiff, UK, Seattle Children's Hospital, Children's Hospital of Eastern Ontario Research Institute in Ottawa. Individuals were diagnosed as having MCAP or MPPH using our published diagnostic criteria[1]. Genomic DNA was extracted from different tissues using standard procedures. When parental DNA samples were available, paternity-maternity testing was performed by genotyping six highly polymorphic short tandem repeats. All exome capture and sequencing experiments were performed using genomic DNA from whole blood.

Case of possible parental mosaicism

LR03-260 is a 9 year-old boy with megalencephaly, hydrocephalus that was shunted at birth, severe bilateral perisylvian PMG (grade 1-2), bilateral postaxial polydactyly of hands and feet, and severe intellectual disability. His occipitofrontal circumference (OFC) was +2 SD above the mean at birth and +5 SD above the mean at 9 years of age. This subject was found to harbor a c.842C>T (p.Pro281Leu) mutation in *CCND2*. His mother's OFC was +2 SD above the mean. She had hypertelorism and borderline intelligence, but no postaxial polydactyly, seizures or hydrocephalus, and had not undergone brain imaging. Sanger sequencing of DNA derived from the mother's saliva and blood was suggestive of her being mosaic for the mutation.

Supplementary Table 1. Phenotypic comparison between *PIK3CA*, *PIK3R2*, *AKT3,* and *CCND2* mutation-positive individuals.

	MPPH			MCAP	CLOVES-FH	HMEG	
Disorder	*PIK3R2*	*AKT3*	*CCND2*	*PIK3CA*	*PIK3CA*	*PIK3CA*	*AKT3*
Type (mosaic vs. germline)	germline	germline	germline	Mosaic, rare germline	mosaic	mosaic	mosaic
Number of reported individuals	13	2	12*	24	16	4	4
Brain overgrowth							
Megalencephaly	13/13	2/2	12/12	24/24	nd	0/4	nd
Hemimegalencephaly	0/13	0/2	0/12	1/24	nd	4/4	4/4
Focal/generalized somatic overgrowth	0/13	2/2	0/12	21/24 (mild-variable)	16/16 (severe)	0/4	nd
Cutaneous – vascular malformations	0/13	0/2	0/12	CapM 24/24	CapM 4/6 VenM 6/6 LymM 4/6 VasM 2/15	0/4	nd
Distal digital anomalies							nd
Postaxial polydactyly	1/13	0/2	11/12	06/24	03/16	nd	nd
Syndactyly	0/13	0/2	0/12	10/24	02/16	nd	nd
Connective tissue dysplasia	0/13	2/2	0/12	19/24	nd	nd	nd
Ventriculomegaly/ hydrocephalus	13/13	2/2	10/12	21/24	nd	2/2	0/4
Cerebellar tonsillar ectopia	8/13	1/2	0/12	20/24	nd	1/2	0/4
Polymicrogyria	13/13	2/2	12/12	19/24	nd	2/2	4/4
Thick corpus callosum	4/13	0/2	1/12	5/24	nd	0/4	0/4
Other abnormalities in CLOVES include wide hands/feet (5/6), sandal gap (4/6), macrodactyly (5/6), paraspinal mass (1/6), asymmetry (5/6), scoliosis (4/6), epidermal nevi (2)							

Abbreviations: CapM, capillary malformations; CLOVES, congenital lipomatous overgrowth, vascular, epidermal, and skeletal; FH, fibroadipose hyperplasia; HMEG, hemimegalencephaly; LymM, lymphatic malformation; MCAP, megalencephaly-capillary malformation syndrome; MPPH, megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndromes; nd, no data; POLY, polydactyly; VasM, vascular malformation; VenM, venous malformations.

*Including the case of possible parental mosaicism described in the Supplementary Note.

Supplementary Table 2. Summary of statistical significance for Figure 4k.

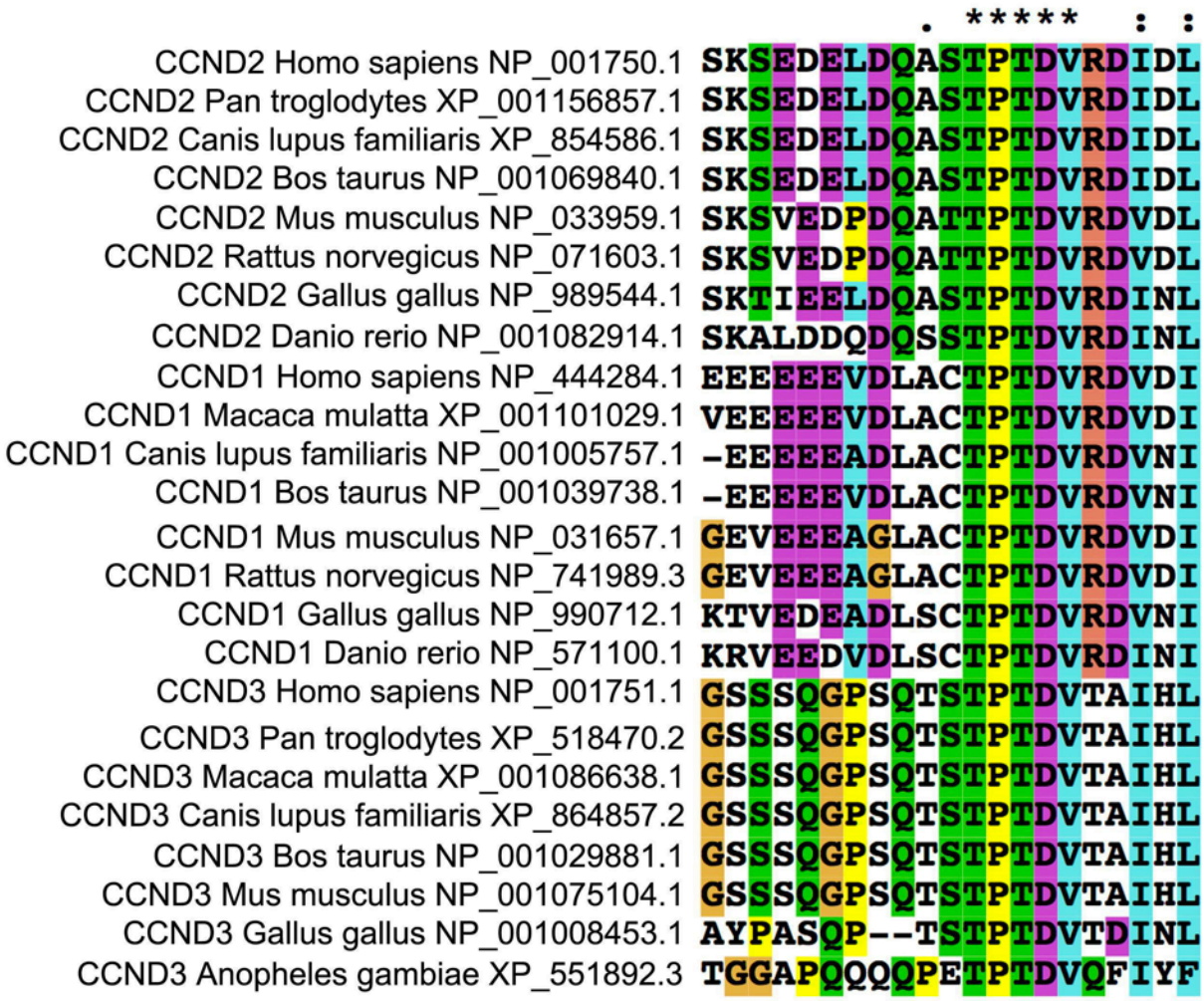
Group	*p*-Value	Group	*p*-Value
GFP vs. CCND2	**0.024**	CCND2 vs. p.Thr280Asp	N.S.
GFP vs. p.Thr280Ala	**<0.0001**	CCND2 vs. p.Pro281Arg	**<0.0001**
GFP vs. p.Thr280Asp	**0.001**	p.Thr280Ala vs. p.Thr280Asp	**0.003**
GFP vs. p.Pro281Arg	**<0.0001**	p.Thr280Ala vs. p.Pro281Arg	N.S.
CCND2 vs. p.Thr280Ala	**<0.0001**	p.Thr280Asp vs. p.Pro281Arg	**<0.0001**

Supplementary Table 3. Summary of statistical significance for Figure 5k.

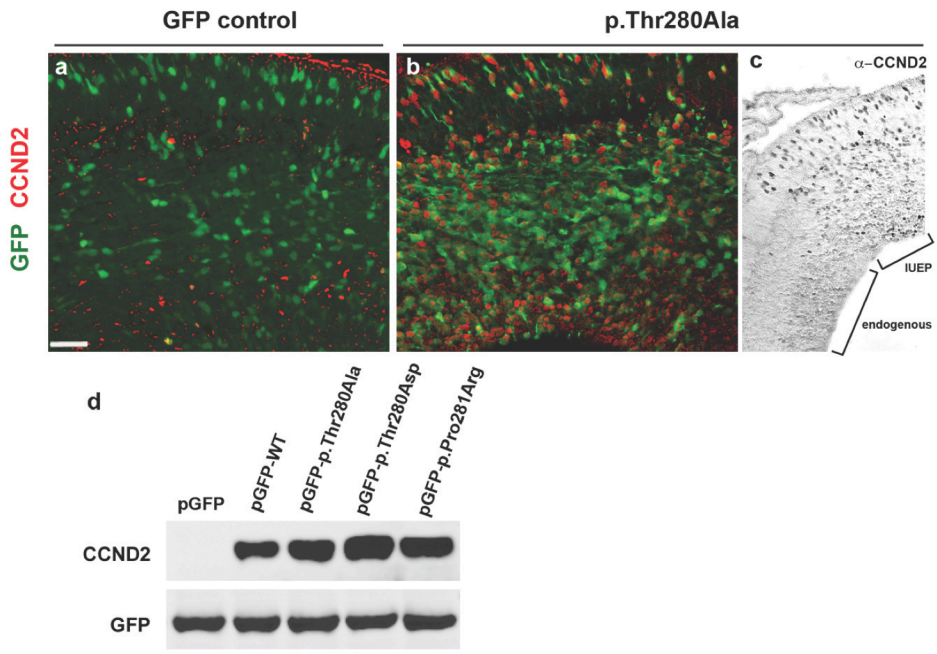
Group	*p*-Value	Group	*p*-Value
CCND2 vs. p.Thr280Ala *P-Fraction*	**0.012**	CCND2 vs. p.Thr280Ala *Exit Fraction*	**0.012**
CCND2 vs. p.Thr280Asp *P-Fraction*	N.S.	CCND2 vs. p.Thr280Asp *Exit Fraction*	N.S.
p.Thr280Ala vs. p.Thr280Asp *P-Fraction*	**0.0071**	p.Thr280Ala vs. p.Thr280Asp *Exit Fraction*	**0.0071**

Supplementary Table 4: Primer pairs for site directed mutagenesis

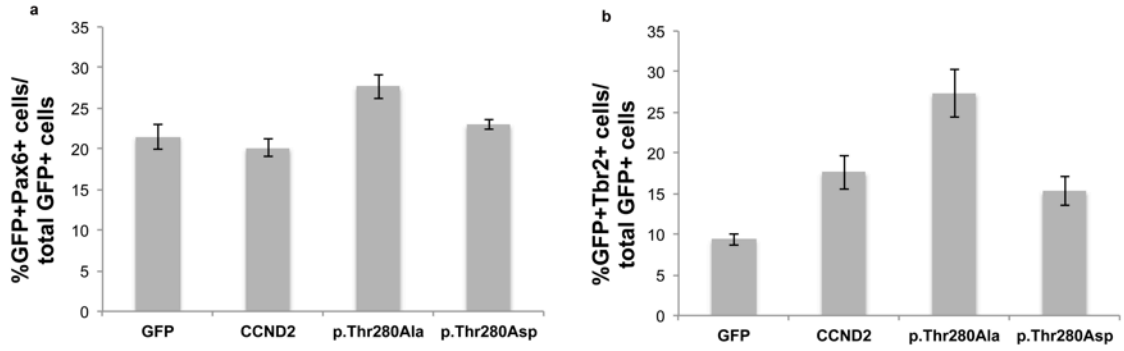
GACCAAGCCAGCGCCCCTACAGACGT /
ACGTCTGTAGGGGCGCTGGCTTGGTC (p.Thr280Ala),
GACCAAGCCAGCACCCGTACAGACGTGCGGG /
CCCGCACGTCTGTACGGGTGCTGGCTTGGTC (p.Pro281Arg),
GCACCCCTACAGACGGGCGGGATATCGACC /
GGTCGATATCCCGCCCGTCTGTAGGGGTGC (p.Val284Gly) and
CAACGTGACGGATCCTAGTCGGAGGATGAACTG /
CAGTTCATCCTCCGACTAGGATCCGTCACGTTG (p.Lys270*).



Supplementary Figure 1. Conservation of the cyclin D protein family C-terminus. Cyclin D family members were identified using Homologene and aligned using ClustalX15. The C-terminal sequence again starting from p.S269 of human CCND2 is shown. Proteins are identified by the corresponding human protein ortholog, species name, and NCBI protein accession identifier. Note the perfect conservation of residues corresponding to human CCND2 positions 280-284 (asterisks).



Supplementary Figure 2. Demonstration of CCND2 protein expression off the IRES-eGFP plasmid constructs. (**a)** Cortical embryonic tissue harvested 48 hours after IUEP with either plasmid expressing only GFP or (**b, c**) plasmid expressing GFP and p.Thr280Ala CCND2. Monoclonal anti-cyclin D2 antibody (anti-CCND2) is best localized in paraffin embedded tissue. Therefore, tissues were processed and embedded in paraffin, sectioned at 4 μm and immunostained with anti- CCND2 and anti-GFP antibodies as in the Methods and previously detailed[2]. (**a**) Two days after IUEP, nearly all of the GFP+ cells are postmitotic and do not co-label with anti-CCND2. (**b, c**) However, 48 hours post-IUEP with plasmid containing mutant *CCND2*, the majority of cells expressing GFP in the cytoplasm also label for CCND2 in the nucleus, including cells that have migrated into the cortical plate. Panel **c** shows a lower magnification that illustrates the difference between endogenous and introduced CCND2. Note that mutant CCND2 persists in cells that have migrated to the cortical plate. The Ki67 labeling in **Fig. 4** indicates that most of these cortical plate cells are non-dividing, implying that CDK inhibitor levels have been induced to push cells into G0. Scale bar=50 μm. (**d)** Western blot of HEK293 cells transfected with the constructs demonstrating CCND2 expression off the IRES-eGFP plasmid. HEK293 cells do not express CCND2 endogenously and therefore, the GFP only transfection shows no CCND2, while all other constructs used in these experiments successfully expressed the exogenous protein.



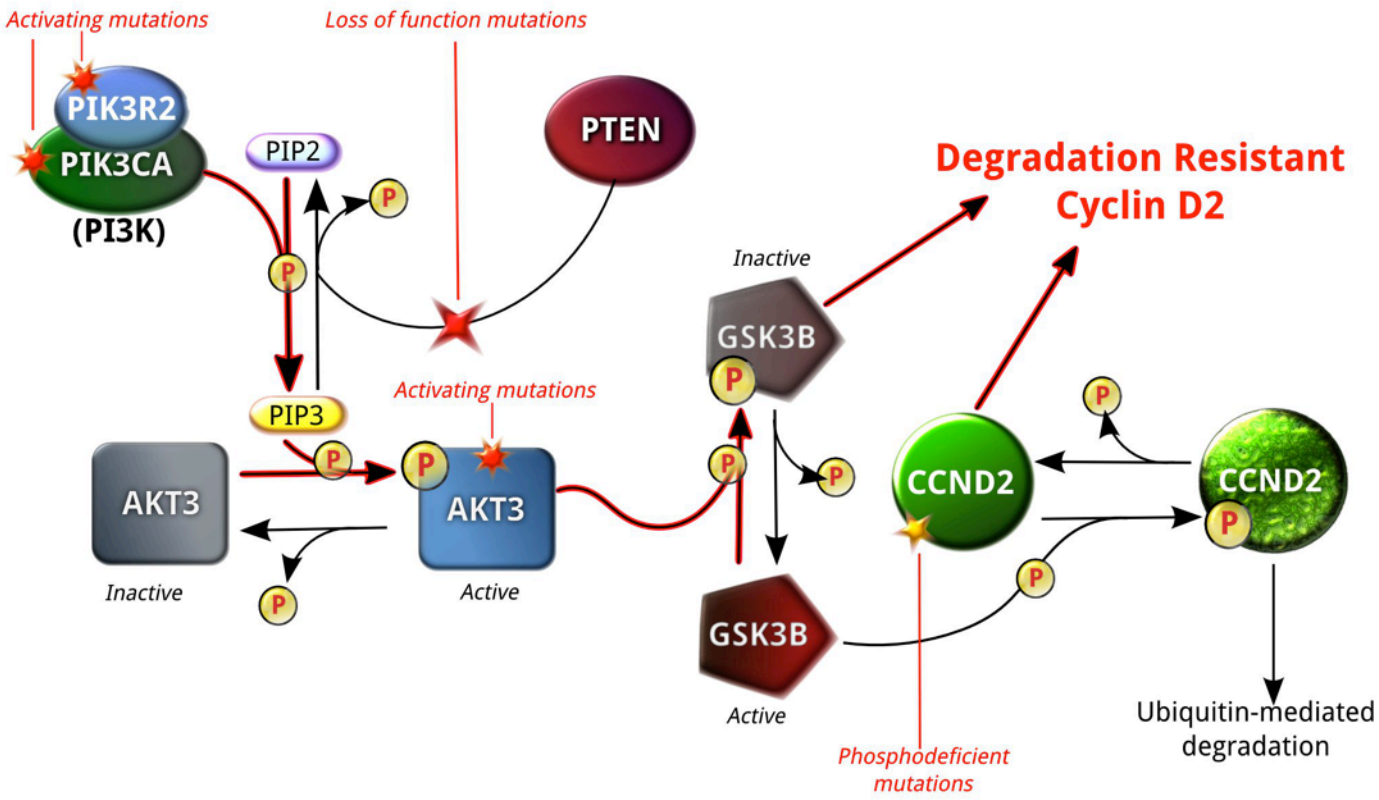
GFP/Pax6

Group	*p*-Value	Group	*p*-Value
GFP vs. CCND2	N.S.	CCND2 vs. p.Thr280Ala	**<0.0001**
GFP vs. p.Thr280Ala	**0.003**	CCND2 vs. p.Thr280Asp	N.S.
GFP vs. p.Thr280Asp	N.S.	p.Thr280Ala vs. p.Thr280Asp	**0.023**

GFP/Tbr2

Group	*p*-Value	Group	*p*-Value
GFP vs. CCND2	**0.006**	CCND2 vs. p.Thr280Ala	**0.002**
GFP vs. p.Thr280Ala	**<0.0001**	CCND2 vs. p.Thr280Asp	N.S.
GFP vs. p.Thr280Asp	**0.046**	p.Thr280Ala vs. p.Thr280Asp	**0.001**

Supplementary Figure 3. Effect of introduced mutant CCND2 on proliferation of (**a**) Pax6- compared with (**b**) Tbr2-expressing progenitors. (**a**) Expression of the phosphodeficient form of CCND2 resulted in an increase in the proportion of electroporated cells that were immunopositive for Pax6 (GFP+Pax6+/ GFP+ total cells), relative to the GFP control, WT CCND2, and phosphomimetic groups. (**b**) Transfection of progenitors with the phosphodeficient form of CCND2 caused a significant increase in the proportion of electroporated cells that expressed Tbr2 compared to the three other groups (GFP, WT CCND2, and the phosphomimetic CCND2). Transfection with the phosphomimetic CCND2 generated results statistically similar to the WT CCND2 sequence. GFP/Pax6 experiment, *n*=6 GFP, *n*=8 WT CCND2, *n*=5 p.Thr280Ala, and *n*=5 p.Thr280Asp. GFP/Tbr2 experiment, *n*=6 GFP, *n*=6 WT CCND2, *n*=5 p.Thr280Ala, and *n*=5 p.Thr280Asp. *n*=embryos, each value average of 5 sections/embryo. Data are presented as mean ± standard error measurement. Significance is tabulated below each graph.



Supplementary Figure 4. Proposed pathway for megalencephaly syndromes. Accumulation of Cyclin D2 (CCND2) may occur either through CCND2 mutations impairing phosphorylation at Thr280 or via mutations in the phosphatidylinositide 3-kinase (PI3K) pathway converging on glycogen synthase kinase 3 β (GSK-3β). Under normal circumstances, GSK-3β phosphorylates CCND2 to target it for ubiquitin-mediated degradation. Constitutively activating mutations of AKT3 result in an increase in phosphorylation of its target GSK-3β, rendering GSK-3β inactive and leading to an accumulation of degradation resistant CCND2. Similarly, mutations in the regulatory (PIK3R2) or catalytic (PIK3CA) subunits of PI3K lead to over-activation of PI3K resulting in increased phosphorylation of phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), activating AKT3 and in turn inactivating GSK-3β. Somatic overgrowth in syndromes such as Cowden syndrome, resulting from loss of function mutations in the phosphatase PTEN, may also result in over-activation of AKT3 and decreased GSK-3β-mediated phosphorylation of CCND2 due to increased PIP3 levels in the absence of dephosphorylation of PIP3 to PIP2 by PTEN.
Arrows highlighted in red show the 'direction' of over-activated pathways in somatic overgrowth syndromes. Phosphate groups are represented by red Ps in yellow circles. The types of mutations resulting in megalencephaly syndromes via apparent accumulation of degradation-resistant CCND2 are marked on the relevant proteins.

References

1 Mirzaa, G. M. *et al.* Megalencephaly-capillary malformation (MCAP) and megalencephaly-polydactyly-polymicrogyria-hydrocephalus (MPPH) syndromes: two closely related disorders of brain overgrowth and abnormal brain and body morphogenesis. *American Journal of Medical Genetics. Part A* **158A**, 269-291, (2012).

2 Glickstein, S. B., Monaghan, J. A., Koeller, H. B., Jones, T. K. & Ross, M. E. Cyclin D2 Is Critical for Intermediate Progenitor Cell Proliferation in the Embryonic Cortex. *The Journal of Neuroscience* **29**, 9614-9624 (2009).