Mice with a conditional deletion of Talpid3 (KIAA0586) – a model for Joubert syndrome

Andrew L Bashford† and Vasanta Subramanian*

Department of Biology and Biochemistry, University of Bath, Bath, UK

*Correspondence to: V Subramanian, Department of Biology and Biochemistry, Building 4 South, University of Bath, Bath, BA2 7AY, UK. E-mail: bssvss@bath.ac.uk
†Current address: Molecular Devices, Amesbury, Wiltshire, UK.

Abstract

Joubert syndrome (JS) is a ciliopathy associated with mutations in numerous genes encoding cilia components. TALPID3 encoded by KIAA0586 in man (2700049A03Rik in mouse) is a centrosomal protein essential for the assembly of primary cilia. Mutations in KIAA0586 have been recently identified in JS patients. Herein, we describe a novel mouse JS model with a conditional deletion of the conserved exons 11–12 of Talpid3 in the central nervous system which recapitulates the complete cerebellar phenotype seen in JS. Talpid3 mutant mice exhibit key hallmarks of JS including progressive ataxia, severely hypoplastic cerebellar hemispheres and vermis, together with abnormal decussation of the superior cerebellar peduncles. The Purkinje cell layer is disorganised with abnormal dendritic arborisation. The external granule layer (EGL) is thinner, lacks primary cilia, and has a reduced level of proliferation. Furthermore, we describe novel cellular defects including ectopic clusters of mature granule neurons, and abnormal parallel fibre-derived synapses and disorientation of cells in the EGL. The defective glial scaffold results in abnormal granule cell migration which manifests as ectopic clusters of granule neurons. In addition, we show a reduction in Wnt7a expression suggesting that defects may arise not only from deficiencies in the Hedgehog (Hh) pathway but also to the additional roles of Talpid3. The Talpid3 conditional knockout mouse is a novel JS model which fully recapitulates the JS cerebellar phenotype. These findings reveal a role for Talpid3 in granule precursor cell migration in the cerebellum (either direct or indirect) which together with defective Hh signalling underlies the JS phenotype. Our findings also illustrate the utility of creating conditional mouse models to assist in unravelling the molecular and cellular mechanisms underlying JS.

Keywords: Joubert syndrome; KIAA0586; primary cilia; cerebellum; sonic hedgehog; ataxia

Introduction

The primary cilium is an antenna-like non-motile structure which protrudes from the cell and consists of a basal body (mother centriole) and an axoneme [1,2]. The components of the sonic hedgehog (Shh) pathway traffic through the primary cilium and transduce signals important for growth, differentiation, and morphogenesis [1,2]. In ciliopathies, cilia are either defective or absent, resulting in multiple organ defects [3–5]. One such ciliopathy is Joubert syndrome (JS) [OMIM (Online Mendelian Inheritance in Man) 21330]. The central diagnostic feature of JS is the ‘molar tooth sign’ seen on MRI of the brain which results from a malorientation of the superior cerebellar peduncles (SCPs). Other brain malformations include hypoplasia of the cerebellar vermis and absence of decussation (both SCPs and the corticospinal tracts at the medullary pyramids) [6–11]. The brain malformations result in the clinical features of ataxia, abnormal eye movements, episodic neonatal breathing abnormalities, and developmental delay. In addition, JS patients can have a wide range of systemic abnormalities including retinitis pigmentosa, renal abnormalities, and polydactyly.

Mutations in around 34 genes have so far been identified as causative for JS (OMIM 21330) and a few of these are components of the basal body. One gene recently identified with mutations in Joubert and Jeune syndrome patients is KIAA0586, also called TALPID3 [12–15]. TALPID3 is a component of the basal body and is present at the distal tip of centrosomes and associates with CP110, Cep97, Kif24, and Cep290 [16]. TALPID3 is conserved in vertebrates [17] and is essential for cilia formation in chicken [18], mice [19], and zebrafish [20].

CEP290 which interacts with TALPID3 is also associated with a variety of ciliopathies with overlapping clinical features including Joubert
syndrome, Joubert syndrome-related disorders (JSRDs) [21], Meckel–Gruber syndrome (MKS) [22], and Bardet–Biedl syndrome (BBS) [23]. The molecular defect in human JS/MKS caused by CEP290 mutations includes proliferation defects of the granule cell precursors (GCPs) associated with abrogated Shh signalling [24].

Constitutive deletion of the highly conserved exons 11 and 12 of Talpid3 in mice results in embryos with a dorsalised neural tube and embryonic lethality at E10.5, while a conditional limb-specific deletion causes polydactyly. Both the dorsalised neural tube and the limb defects are due to abrogation of Shh signalling [19]. Here, we describe the phenotype of mice with a conditional deletion of the conserved exons 11 and 12 of Talpid3 in the brain which resembles the human JS phenotype.

Materials and methods

Study approval

All animal procedures were reviewed and approved by the Animal Welfare and Ethical Review Body of the University of Bath and conducted under approved Project and Personal licences in accordance with UK Home Office guidelines and the UK Animals (Scientific Procedures) Act, 1986.

Mouse strains and breeding

Talpid3<sup>flo/flo</sup> mice [19] were maintained on a 12 h light/dark cycle with access to food and water ad libitum. CNS specific recombination was achieved by crossing the Talpid3<sup>flo/flo</sup> mice with the NestinCre line [B6.Cg-Tg(Nes-Cre)1Kln/J] [25]. Offspring were genotyped by PCR for the Cre transgene and the floxed and deleted Talpid3 alleles [19].

Histology

Postnatal mice were euthanised by injection of sodium pentobarbitone (200 mg/kg; Euthatal, Boehringer Ingelheim Animal Health, Bracknell, UK). For cell proliferation studies, mice were injected with bromodeoxyuridine (BrdU; 100 mg/kg body weight; Sigma-Aldrich Co Ltd, Gillingham, UK) 1 h before euthanasia. Dissected brains were fixed overnight at 4°C in 4% buffered paraformaldehyde (Sigma-Aldrich Co Ltd), embedded in paraffin wax (Fibrowax™, VWR, Leuven, Belgium) or frozen in OCT (Tissue-Tek<sup>®</sup>, Sakura, Japan), sectioned, and immunostained.

Immunohistochemistry

Frozen sections were rinsed in PBS to remove OCT. Paraffin sections were dewaxed in Histoclear (National Diagnostics, Yorkshire, UK) and rehydrated. Antibodies were blocked in blocking buffer (PBS with 0.1% gelatin, 0.5% BSA, and 0.1% Tween-20) for 1–2 h. Sections were incubated overnight at 4°C with the following primary antibodies: mouse anti-calcibindin (1:4000, 300; Swant, Marly, Switzerland); mouse anti-neurofilament (1:5, 2H3; DSHB, Iowa City, IA, USA); rabbit anti-pericentrin (1:2000, 448; Abcam, Cambridge, UK); rabbit anti-adenyl cyclase III (1:1000, C-20; Santa Cruz, Dallas, TX, USA); rabbit anti-active caspase 3 (1:2000, ab13847; Abcam); rabbit anti-Pax6 (1:500, PRB-278P; Covance, Dedham, MA, USA); rabbit anti-phosphohistone 3 (1:1600, 3377; Cell Signaling Technology, Danvers, MA, USA); guinea pig anti-VGlut (1:6000, Ab5905; Millipore, Billerica, MA, USA); rabbit anti-GAD65/67 (1:500, AB1511; Millipore, Watford, UK); and mouse anti-PCNA (1:4000, 2586; Cell Signaling Technology).

Sections were treated with pepsin (0.1% pepsin in 0.1 M HCl; Sigma-Aldrich Co Ltd) for the detection of BrdU, blocked in blocking buffer, and incubated with the following primary antibody: mouse anti-BrdU (1:50, G3G4; DSHB) overnight at 4°C.

Unbound primary antibody was washed off in PBST (3 x 10 min) and the appropriate secondary antibody was applied to the sections and incubated for 2 h at room temperature. Secondary antibodies used for indirect immunofluorescence were rabbit anti-mouse Alexa 568 (A11061), goat anti-guinea pig Alexa 488 (A-11073), and goat anti-rabbit Alexa 488 (A-11034), all at 1:1000 dilution (Invitrogen, Waltham, MA, USA). Images were acquired using a Leica DM555 microscope [Leica Microsystems (UK) Ltd, Milton Keynes, UK], a DFC-6000 camera [Leica Microsystems (UK) Ltd], and LAS software [Leica Microsystems (UK) Ltd]. Image analysis was performed using Fiji software [26].

RNA isolation, cDNA synthesis, and RT-qPCR analysis of signalling pathway components

Cerebella were snap-frozen in Trizol reagent (Invitrogen) and total RNA was extracted. cDNA synthesis was performed using RevertAid H-Minus Reverse Transcriptase (Thermo Fisher Scientific, Loughborough, UK). Quantitative PCR was carried out in an IQ5 thermocycler (Bio-Rad Laboratories Inc, Watford, UK) using SYBR Green Supermix (Bio-Rad Laboratories Inc). Expression levels were quantified relative to Gapdh and comparisons were made between control and mutant mice (n = 3 or 4). Student’s t-test was used to compare mean expression values between control and mutant mice. Primer sequences and product size are listed in supplementary material, Table S1.

Western blotting

Cerebella were minced and homogenised in lysis buffer (2% SDS in 50 mM Tris, pH 6.8) and cleared lysates were snap-frozen. Protein concentration was estimated using a BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific) and proteins were resolved on a 7%
Tris–glycine SDS gel (National Diagnostics) and then transferred to a PVDF membrane (Millipore). Blots were incubated overnight at 4 °C with the appropriate primary and secondary antibodies and binding was detected using the ECL chemiluminescence reaction (Pierce, Thermo Fisher Scientific) and a Vilber Lourmat Fusion imager (Collégien, France).

Details of all methods including image analysis, quantification, and statistical analysis are provided in supplementary material, Supplementary materials and methods.

Results

Deletion strategy and gross phenotype of Talpid3 mutant mice

We conditionally deleted the conserved exons 11 and 12 of Talpid3 in the developing nervous system by crossing mice homozygous for the floxed Talpid3 allele (Talpid3fl/fl) to the Nestin::Cre (NesCre) deleter strain [25] (Figure 1A,B).

Since both the Talpid3 gene and the NesCre transgene are located on mouse chromosome 12, we obtained a reduced frequency of mice homozygous for the Talpid3fl/fl allele carrying the NesCre transgene (Talpid3fl/flNesCre). Throughout this paper, we will refer to the Talpid3fl/flNesCre mice as the Talpid3 (Ta3) mutant mice and the Talpid3fl/fl littermates as wild type. It is noteworthy that all the Talpid3 mutant mice that we examined consistently exhibited the phenotype that we describe here including the ataxia.

The Talpid3 mutant mice were visually indistinguishable from their wild-type littermates at birth, but by P15 they were smaller with a pronounced domed head (Figure 1C). The first signs of ataxia appeared at P10; ataxia progressed rapidly and was very severe by P15 (supplementary material, Video S1). However, the Talpid3 mutant mice were still able to suckle as well as groom themselves (supplementary material, Video S1).

Macroscopic examination of the brains of P15 Talpid3 mutant mice revealed a markedly hypoplastic cerebellum with a significantly smaller vermis and cerebellar hemispheres compared with the wild type (Figure 1D,E). These morphological abnormalities resemble the phenotype of JNS/MS patients who have cerebellar hypoplasia and a markedly small or absent vermis [6,8]. The P15 Talpid3 mutant brain also exhibited hydrocephaly, which was first detectable at P5 and progressed in severity between P5 and P15, forcing the superior and inferior colliculi into a more posterior position (Figure 1F). The hydrocephaly is unlikely to have a direct bearing on the cellular defects seen in the cerebellum as the proliferation and migration defects are already observable by P0, much before the development of hydrocephaly.

We corroborated the gross morphological changes seen in the brains of the Talpid3 mutant mice with histological analysis focusing on the cerebellum. We analysed growth and fissure formation, as well as the effects on decussation of the SCPs. The five cardinal lobes of the cerebellum (anterobasal, anterodorsal, central, posterior, and inferior) and the four principal fissures arise first during the postnatal development of the cerebellum [27,28]. In contrast to the normal developmental sequence of the cardinal lobes and fissure formation seen in wild-type mice, the Talpid3 mutant cerebella showed a dramatic loss of foliation which was most severe in lateral regions (supplementary material, Figure S1A–E). The formation of the cardinal lobes in Talpid3 mutant cerebella was retarded. This was first evident at E18.5, when fissures were barely visible (supplementary material, Figure S1A). There was no significant increase in size or foliation in the Talpid3 mutant cerebella between E18.5 and P10 (supplementary material, Figure S1A–C). At P15, only the five cardinal lobes were present in the Talpid3 mutant cerebellum and the fissures remained shallow (supplementary material, Figure S1E), suggesting a defect in proliferation and growth. The cross-sectional area of the vermis was significantly reduced in the Talpid3 mutant cerebellum (Figure 2A,B).

The most striking feature of the Talpid3 mutant cerebellum at P5 was the thin external granule layer (EGL), which had a lower cell density compared with the wild type (supplementary material, Figure S2A,B), and the inner granule layer (IGL) at P15 (Figure 2C,D and supplementary material, Figure S2A,D), which was sparsely populated with cells (Figure 2C,D). The integrity of the molecular layer (ML) and the IGL was lost in the P15 Talpid3 mutant cerebella and the discrete single-cell Purkinje cell (PC) layer was disrupted (Figure 2C,D).

We further assessed the organisation of the midbrain, the hindbrain, and the SCPs by immunostaining for neurofilament M in horizontal sections. The midbrain appeared thinner across the medio-lateral axis (Figure 2E,F). Assessment of serial sections across the dorso-ventral axis identified comparable regions but these were present at different axial levels due to the very large difference in shape and size of the brain. The gross organisation of the tracts in the midbrain appeared to be compressed and misshapen (Figure 2F), and the decussation of the SCPs was disrupted in the Talpid3 mutant brain (Figure 2E,G).

Cerebella of Talpid3 mutant mice lack primary cilia

It has previously been shown that the deletion of the coiled-coil domain of Talpid3 is sufficient to disrupt cilia formation by the failure of the mother centriole to dock with the plasma membrane [18,19]. We analysed the cerebella of Talpid3 mutant mice for primary cilia by immunostaining for adenylyl cyclase III (ACIII), a protein shown to localise at primary cilia in neurons and glial cells [29].

Primary cilia were present in the majority of cells in the EGL and IGL in wild-type mice (supplementary material, Figure S3A,C). In contrast, cells in the
Figure 1. Talpid3 knockout strategy and gross phenotype of the brain. Strategy for deletion of the coiled coil domain encoded by exons 11 and 12. The Talpid3<sup>fl/fl</sup> mice in which exons 11 and 12 are flanked by LoxP sequences were crossed to the NesCre deleter mouse, leading to the deletion of these exons. (B) PCR amplicons used to identify mice with wild-type and Talpid3<sup>fl/fl</sup> alleles and the NesCre transgene. (C) P15 Talpid3 mutant mice are smaller with a domed head (arrow). (D) Posterior view of P15 brain; Talpid3 mutants have hydrocephaly and hypoplasia of the cerebellum. Talpid3 mutant cerebrum appears slightly collapsed due to severe hydrocephaly indicated by the dashed blue line. (E) Lateral view of P15 whole-mount brains and (F) sagittal sections of brain stained with H&E. Upper panel: wild type; lower panel: Talpid3 mutant. Note the severe hydrocephaly (asterisk), posterior shift in colliculi (white arrow), and hypoplasia of the cerebellum (black arrow) in the lower panel of F. Coloured regions indicate main brain structures: cerebellum (orange), cerebrum (blue), colliculi (green), hindbrain (yellow), olfactory bulb (magenta). Scale bars: 10 mm (C); 3 mm (D, E); 2 mm (F).

cerebella of Talpid3 mutant mice had no cilia or showed fine dots staining positive for ACIII but importantly these did not resemble primary cilia (supplementary material, Figure S3A,C). In the wild-type EGL, 64.4% of cells possessed fully formed primary cilia, while in the mutant, 18.7% of the cells appeared to only have fine dots staining positive (supplementary material, Figure S3B). In the wild-type IGL, fully formed primary cilia were present on 60.9% of cells compared with less than 10% of cells with dot-like ACIII-stained structures in the Talpid3 mutant (supplementary material, Figure S3D).

Effect of loss of Talpid3 on GCPs and proliferation
GCPs originate from the rhombic lip and migrate to form the EGL of the cerebellum. The extensive...
Figure 2. Histology of the cerebella of *Talpid3* mutant and wild-type mice. (A) H&E-stained cerebellar sections of wild-type and *Talpid3* P15 mice. Note haematoma in the posterior cerebellar lobe (white arrow). (B) Cross-sectional area of cerebellar vermis showing reduction in the *Talpid3* mutant. (C, D) Cerebellar lobe showing reduced granule neurone density in the IGL and a disorganised PCL with ectopic PCs (black arrows); some PCs appear pyknotic. *Talpid3* mutant ML also shows clusters of cells at P15 which are absent in the wild-type ML. (E) P15 horizontal section of wild-type and *Talpid3* mutant brain stained for neurofilament. Note the thinner and elongated midbrain of mutant mice and hydrocephaly of the lateral ventricles. (F) Greater magnification of the boxed region of the midbrain in E. Note the reduced density of tracts in the superior cerebellar peduncles, indicated by an asterisk. (G) Abnormal decussation of cerebellar peduncles with compact and thickened tracts, indicated by 'X'. Greater magnification of the boxed region from F. Sagittal image shows the dorso-ventral position of horizontal sections. 3PC, parvocellular oculomotor nucleus; 3V, third ventricle; 4V, fourth ventricle; ac, anterior commissure; f, fornix; fr, habenulo-interpeduncular tract; ic, internal capsule; OB, olfactory bulb; poDG, posterior dentate gyrus; scp, superior cerebellar peduncle; xscp, decussation of the superior cerebellar peduncle. Anatomical structures were identified using The Allen Mouse Brain Library C57BL/6J horizontal atlas. Scale bars: 500 μm (A, C); 50 μm (D); 1 mm (E, F); 100 μm (G). Note: Fiji software was used to 'stitch' large composite pictures from overlapping images (A, E, F). Sequential images of equal exposure were taken in a grid across the region of interest with adjacent images having a small overlap. The Fiji software aligned the identical overlapping regions to stitch images together.
proliferation of GCPs in the EGL is dependent on Shh signalling [30–35]. Post-mitotic cells migrate tangentially and then radially inwards to the IGL. Since the Talpid3-mutant EGL cells lacked primary cilia, we investigated the proliferation of GCPs in the Talpid3 mutant mice and compared it with their wild-type littermates in 18.5 dpc (days post coitum) embryonic and postnatal cerebellum.

GCP proliferation was assessed at E18.5, when the immature cerebellum begins its massive growth and organisation [27,28]. Cerebellar sections were stained for phosphohistone3 (PH3) and proliferating cell nuclear antigen (PCNA), and proliferating cells in each of the emerging cardinal lobes were quantified. There was a significant reduction in the number of PH3+ cells in four out of five regions and a reduction in PCNA+ cells in all regions (supplementary material, Figure S4).

We observed significantly fewer granule cells in the postnatal cerebella of Talpid3 mutant mice and to assess if this was due to defective proliferation, we administered the thymidine analogue bromodeoxyuridine (BrdU) to Talpid3 mutant and wild-type sibling mice at P5, P10, and P15. Immunohistochemistry for BrdU and PH3 identified cycling cells in S-phase or mitosis, respectively (Figure 3A). In the Talpid3 mutant cerebella, there was a significant reduction in the number of proliferating cells in the EGL in all lobes at P5 (Figure 3B,C) and P10. By P15, proliferation was considerably reduced in both wild-type and Talpid3 mutant cerebella such that relative differences in proliferation observed earlier were not obvious (Figure 3B,C). The thickness of the EGL in the Talpid3 mutant cerebellum was significantly reduced, with a lower density of cells per unit length (Figure 3C).

Concomitant with defective GCP proliferation, there was a noticeable reduction in the density of granule cells in the IGL. On average, P5 Talpid3 mutants exhibited a 27% reduction in cell density in the IGL (Figure 3D). To assess whether the lower cell density was due to a combination of reduced cell proliferation and increased apoptosis, we immunostained cerebellar sections for cleaved caspase-3 (supplementary material, Figure S5A,B). Talpid3 mutants had an increased number of apoptotic cells per unit area in the EGL (supplementary material, Figure S5C) but not in the IGL (supplementary material, Figure S5D).

Orientation and migration of GCPs

In addition to reduced proliferation and increased apoptosis, we also observed that the GCPs appeared more elliptical and were tangentially orientated in the Talpid3 mutant EGL (supplementary material, Figure S6A). We analysed and quantified the nuclear orientation of proliferating cells in the EGL in all lobes at P5 (Figure 3B,C) and P10. By P15, proliferation was considerably reduced in both wild-type and Talpid3 mutant cerebella such that relative differences in proliferation observed earlier were not obvious (Figure 3B,C). The thickness of the EGL in the Talpid3 mutant cerebellum was significantly reduced, with a lower density of cells per unit length (Figure 3C).

Concomitant with defective GCP proliferation, there was a noticeable reduction in the density of granule cells in the IGL. On average, P5 Talpid3 mutants exhibited a 27% reduction in cell density in the IGL (Figure 3D). To assess whether the lower cell density was due to a combination of reduced cell proliferation and increased apoptosis, we immunostained cerebellar sections for cleaved caspase-3 (supplementary material, Figure S5A,B). Talpid3 mutants had an increased number of apoptotic cells per unit area in the EGL (supplementary material, Figure S5C) but not in the IGL (supplementary material, Figure S5D).

Orientation and migration of GCPs

In addition to reduced proliferation and increased apoptosis, we also observed that the GCPs appeared more elliptical and were tangentially orientated in the Talpid3 mutant EGL (supplementary material, Figure S6A). We analysed and quantified the nuclear orientation
and nuclear area of cells in both Talpid3 mutant and wild-type EGL. In the P5 EGL of wild-type mice, nuclei tended to orientate in a radial direction and this shifted to a tangential orientation in the granule cell-depleted EGL by P15 (supplementary material, Figure S6B). In the P5 Talpid3 mutant cerebellum however, EGL cells were mostly tangentially orientated and remained so until P15 (supplementary material, Figure S6B). Measurement of the nuclear area also showed that in the wild-type EGL, the nuclei were more compact with a smaller median area (supplementary material, Figure S6C).

The Talpid3 mutant ML contained significantly large numbers of ectopic cells and its boundaries with the EGL and IGL were less defined. We identified ectopic cells by co-immunostaining for Pax6 and the mature neuron marker NeuN. In wild-type cerebella, GCPs in the EGL and migrating cells in the ML strongly express Pax6 and the IGL cells are negative (Figure 4A). Immature cells of the EGL and ML express very low levels of NeuN, which becomes stronger as they mature in the IGL. In the Talpid3 mutant ML, however, we saw clusters of cells strongly expressing NeuN with barely detectable to low levels of Pax6, indicating that they were maturing/mature granule neurons (Figure 4A). It is likely that these ectopic NeuN-positive cells have undergone premature maturation in the ML before reaching the IGL due to defective migration.

To further characterise the organisation of the ML, we stained for the 165 kDa neurofilament M, which identifies axonal tracts. At P5, the organisation of the axonal tracts appeared similar between wild-type and Talpid3 mutant, with long axons originating from the central nuclei extending radially through the IGL but which were denser in the Talpid3 mutant cerebella (Figure 4B). By P10, the axonal tracts in the ML of the wild-type cerebellum (Figure 4B) extended both tangentially and radially in the basal half of the ML. In contrast, the axonal tracts in the Talpid3 mutant ML extended predominantly in the radial axis, which became more pronounced by P15 in the Talpid3 mutant (Figure 4B).

Glia scaffold defects

Bergmann glia form the scaffold on which the granule neurons migrate to the IGL. To assess the morphology of the glial scaffold, we stained sections of the cerebellum for GFAP at P5, P10 and P15. In wild-type cerebella, the glial scaffold consisted of parallel well-organised fibres which increased in density by P15 (Figure 4C,D). In the Talpid3 mutant cerebella, there was a reduction in the number of Bergmann glia and a concomitant loss in the density of glial fibres extending through the ML (Figure 4C,D). The Talpid3 mutant glial scaffold was not only less dense but also disorganised, with more tangentially orientated fibres (Figure 4C,D).

Bergmann glia were further identified by immunostaining their radial fibres for nestin and their cell soma with BLBP (supplementary material, Figure S7). In the wild-type P5 cerebellum, the position of the cell soma was in the boundary between the IGL and ML, whereas in the Talpid3 mutant, numerous cell bodies were mislocalised throughout the ML (supplementary material, Figure S7A) and this was more prominent by P15 (supplementary material, Figure S7B). Interestingly, the misorientation of granule cell nuclei correlated with an increased number of tangentially orientated glial fibres in the E. Double labelling for the granule precursor marker Pax6 and the glial protein GFAP at P10 clearly showed neat parallel glial fibres in the wild-type EGL but the Talpid3 mutants exhibited an aberrant organisation with more tangential fibres lying adjacent to tangentially orientated Pax6-positive GCPs (Figure 4E).

The Purkinje cell layer is disorganised with abnormal PCs

In wild-type mouse cerebella, the PCs start expressing Shh when they integrate into the PCL and this acts as a potent mitogen leading to massive proliferation of the GCPs [30–35]. In H&E-stained sections of the wild-type cerebellum, the PCs formed a distinct monolayer surrounding the internal granule layer (IGL), which was disorganised in the Talpid3 mutant cerebellum with heterotopic PCs (Figure 2D). To investigate this further, we stained PCs with an antibody to calbindin D-28k [36], a calcium binding protein, which confirmed the presence of a disorganised PCL. In addition in the Talpid3 mutant, groups of PCs were present at the base of the folds as well as in the centre of the IGL and ML (supplementary material, Figure S8A,B).

The Talpid3 mutant cerebella, although smaller than the wild type, had a surprisingly high density of PCs. The Talpid3 mutant P10 cerebellum had a 23% reduction in the total number of PCs but a density approximately twice that of wild type (supplementary material, Figure S8C,D). Since there was an apparent loss of PCs in the Talpid3 mutant mice, we investigated if the reduction in PC numbers was due to increased cell death. Staining for the apoptotic marker cleaved caspase-3 together with calbindin D-28k did not detect any apoptotic PCs in the wild-type or mutant cerebella at P10 (supplementary material, Figure S3A).

The dendritic arborisation of the PCs in the Talpid3 mutant cerebella exhibited asymmetry and disorganisation. The primary dendrite that extended from the cell body to the first bifurcation branched prematurely (supplementary material, Figure S8E–G). In addition, a small number of PCs appeared to be completely misorientated, extending processes in the wrong direction (supplementary material, Figure S8E). This correlated with the loss of the discrete layering in the cerebellar cortex and the lack of postnatal growth. In the wild-type cerebellum, the PCs displayed a characteristic pattern of dendrites with a clear primary branch which bifurcated to eventually form the numerous spiny branches at the distal tips. In the Talpid3 mutant cerebella, the PCs had a shorter primary dendrite (supplementary material, Figure S8E–H) which branched into fine structures...
Figure 4. Glial scaffold and migration of GCPs in Talpid3 mutant and wild-type cerebella. (A) P10 wild-type and Talpid3 mutant cerebella immunostained for NeuN and Pax6. Arrows indicate ectopic NeuN+ cells in the molecular layer. The boxed region indicates enlarged area showing ectopic NeuN+ cells in the molecular layer. (B) P5, P10, and P15 cerebella immunostained for 165 kDa Nf. (C) P5, P10, and P15 cerebella labelled for GFAP. (D) Outline of P15 glial fibres immunostained for GFAP. (E) P10 cerebella immunostained for Pax6 and GFAP. Arrows indicate tangentially orientated glial fibres in the EGL. EGL, external granule layer; GFAP, glial fibrillary acidic protein; IGL, internal granule layer; ML, molecular layer; Nf, neurofilament. Scale bars: 50 μm.

more similar to the spiny distal tips (supplementary material, Figure S8E,G).

Effects of Talpid3 loss on cerebellar circuits
The cerebellum modifies and coordinates motor signals and undergoes the majority of its growth postnatally as animals learn to move [27,28] and PCs constitute the principal neuron in cerebellar circuits. Granule neurons, the presynaptic afferents of the PCs, have previously been shown to be potent regulators of PC development [37]. Since Talpid3 mutants are ataxic and we saw aberrations in GCP proliferation and organisation of the ML, IGL, the PCs, and their dendrites, we investigated if there were defects in cerebellar circuit formation. We visualised the different subsets of nerve terminals on
the dendrites of PCs by immunostaining with antibodies directed against distinct nerve terminal-associated proteins.

Glutamic acid decarboxylase (GAD-65/67), the enzyme responsible for GABA synthesis, is a marker for presynaptic inhibitory terminals originating from stellate and basket interneurons. Inhibitory terminals were identified by labelling with antibodies against GAD-65/67. In both wild-type and Talpid3 mutant PCs, GAD-65/67-positive synapses were present throughout the dendritic arbors (Figure 5A,B) and the overall density and distribution of inhibitory synapses were comparable.

Excitatory synapses formed by the climbing fibres and granule cell parallel fibres (PFs) were labelled with antibodies against vesicular glutamate transporter 1 (VGlut1) [38]. In the cerebella of wild-type mice, the excitatory synapses were clustered at primary dendrites of the PCs but in the Talpid3 mutant this was disrupted and excitatory synapses were seen throughout the dendritic arbors extending into the ML as well as the EGL (Figure 5C,D). This suggests that the cerebellar circuitry is significantly disrupted in the Talpid3 mutant mice.

Signalling pathways in Talpid3 mutant mice

Primary cilia are known to be essential for hedgehog signalling [34,35] and it has been shown by Aguilar et al that levels of GLI1 and PTCH1 mRNA are significantly reduced in most cases of JS [24]. We assessed the effects of loss of Talpid3 and cilia on Shh pathway components in the cerebella of Talpid3 mutant mice by RT-qPCR. The expression levels of Gli1 and Ptc1, the target genes of the Hh pathway in the GCPs, were significantly reduced in Talpid3 mutant mice (Figure 6A).

We also investigated the expression levels of the components of the Notch signalling pathway (Notch1, Notch2, Dll1, Dll3) and Notch-responsive genes (Hes1, Hes5) by RT-qPCR, but no significant differences were observed between Talpid3 mutant and wild-type siblings (Figure 6B). The Wnt signalling pathway has been suggested to play an important role in cerebellar development. Since we see aberrations in cerebellar patterning in the Talpid3 mutant mice, we analysed the expression of Wnt3a and the Wnt-responsive gene Axin2 but found no significant changes in their expression levels compared with wild-type sibling mice. However, we did see a considerable reduction in the expression of Wnt7a (Figure 6C) in the Talpid3 mutant cerebella.

To further assess the defect in Shh signalling, processing of both Gli2 and Gli3 proteins was analysed by western blotting of cerebellar extracts from Talpid3 mutant and wild-type sibling mice. Analysis of Gli2 protein detected two high-molecular-weight bands at 179 and 169 kDa and a lower band at 89 kDa, which correspond to Gli2 activator and Gli2 repressor, respectively, in the wild-type mice. The Talpid3 mutant mice showed a consistent reduction in Gli2A bands (Figure 6D) when compared with the wild-type sibling.

Analysis of Gli3 protein showed similar results; in all wild-type sibling mice, a faint band was observed at 185 kDa and one at 93 kDa, corresponding to Gli3 activator and Gli3 repressor, respectively. A non-specific band was also seen at 142 kDa, which is consistent with previous studies using the anti-Gli3 antibody [39]. The
level of Gli3A was low in mice of both genotypes; however, in the cerebella of Talpid3 mutant mice, there was a noticeable increase in the level of Gli3R (Figure 6E).

Discussion

We created a CNS-specific conditional knockout mouse for Talpid3 since the constitutive Talpid3 mutant mouse is embryonic lethal (E9.5–10.5) [19]. We investigated if its phenotype resembled JS as mutations in Talpid3 have been identified in JS patients. Deletion in Talpid3 leads to a loss of primary cilia, which are essential for Shh signalling (refs 18–20 and this study). Here, we show for the first time that a conditional deletion in Talpid3 in the CNS of mice recapitulates the human JS phenotype such as ataxia, hypoplastic cerebellar hemispheres, small vermis, and abnormal decus- sation of the SCPs. A limb-specific knockout described in a previous study has polydactyly [19], another feature which is sometimes found, although not essential for the diagnosis of JS.

Mouse mutants have been generated for a few JS-causative genes such as Cep290, Ahi1, Cep41, Tmem67, Rpgr, and Cep120 [40–46]. Most of these mice are constitutive knockouts or gene-trapped. The
phenotypes reported for some of these mutants are at embryonic stages (when the cerebellum is either not developed or fully formed) and in other cases do not fully manifest all the features of JS [40–46]. Unlike the conditional Talpid3 mutant mice, the cerebellum was not severely affected in the Ah1 and Cep290 mutant mice described by Lancaster et al [40]. The brain in the Ah1 mutants was slightly smaller with mild foliation defects in the cerebellum, whereas in the Cep290 mutants, only foliation was mildly affected. In addition, both mutant mice had midline fusion defects, which in the case of the Ah1 mutant were attributed to defective Wnt signalling [40]. In another mouse mutant for Cep290 generated by gene trapping, the cerebellum appeared normal but the brain developed hydrocephaly [41].

The Talpid3 mutant mice develop many of the histological and cellular features of human JS such as loss of integrity of the PCL, presence of heterotopic PCs, and ectopic mature neurons in the ML [24]. At the molecular level, there is a significant reduction in the expression levels of Gli1 and Ptc1 in the Talpid3 mutant cerebella and defective processing of Gli2 and Gli3 with aberrant Shh signalling similar to that seen in human JS/MKS cerebella [24]. The severe cerebellar phenotype seen in the Talpid3 mutant can be attributed to an increase in Gli2 and Gli3 repressors. This is in contrast to the Cep290 and Ah1 mutant mice [9,40] in which defects in GCP proliferation or effects on Shh pathway components were not detected in later stages of hindbrain development.

A conditional mouse mutant for the Talpid3 interacting protein Cep120 has been reported which lacks primary cilia and has a hypoplastic cerebellum with reduced GCP proliferation [46]. However, the Purkinje cell layer does form normally, unlike in the Talpid3 mutant. In addition, loss of Cep120 caused centriole duplication, which we did not observe in the Talpid3 mutant. Mouse mutants of the intraflagellar transport proteins Kif3a and Ift88 exhibit defective GCP proliferation, a disrupted PCL, and aberrant development of Bergmann glia resulting from the inability to respond to Shh signalling [34,35], similar to that seen in the Talpid3 mutant. The loss of Kif3a results in a significant reduction in Gli1 expression, also seen in the Talpid3 mutant [34].

Besides Shh, other signalling pathways have also been suggested to act through primary cilia and include the Wnt and Notch pathways [10,47]. Wnt signalling is important in early cerebellar development; however, in the Talpid3 mutant cerebella there was no significant effect on the expression levels of Axin2, a well-known negative regulator of Wnt signalling which is upregulated upon activation of the canonical Wnt pathway [48]. Interestingly, we did observe a reduction in Wnt7a expression in the Talpid3 mutant cerebella. Wnt7a is implicated in granule cell development. It is expressed postnatally in the IGL and in cell cultures has been shown to play a role in axonal migration and branching [49]. It is tempting to speculate that the reduction in Wnt7a expression levels seen in the mutant cerebellum may underlie the maturation and migration defects seen in the granule neurons. Another possible explanation for the reduction in Wnt7a expression is that the loss of granule neurons results in a smaller number of cells expressing Wnt7a, thus affecting the expression levels. At present, it is unclear whether the effect on Wnt7a expression is a cause or a consequence of the deletion of Talpid3 exons 11–12.

In addition to the reduction in GCP proliferation caused by the loss of primary cilia and Shh signalling, a consistent defect seen in the Talpid3 mutant cerebella was the aberrant migration of GCPs. This manifested as misoriented GCPs, many of which failed to reach the IGL. These migration defects coincide with an aberrant scaffold formed by the Bergmann glia. Migration defects of talpid3 mutant cells were first described in an explant culture of chick limb mesenchymal cells [50]. The saltatory migration of talpid3 mutant cells had greater periods of rest rather than a defective rate of migration. Yin et al found that talpid3 mutant cells in culture showed slower microtubule regrowth [18], which may explain the migration defects. Presumably, this could be due to the role of the centriole as a microtubule-organising centre in the cell. A scenario is possible in which slow-moving cells simply become trapped in the rapidly developing matrix, and this might explain the migration defects that result in the ectopic cells seen in the Talpid3 mutant ML. This defect may be exacerbated by the defective glial scaffold. Stephen et al [12] have also shown polarity defects in the neuroepithelium of the talpid3 chick, suggesting that Talpid3 has other functions in addition to its role in cilia formation.

The Talpid3 constitutive knockout mouse is embryonic lethal (mice survive until E10.5) [19] but mice with conditional deletion of exons 11 and 12 in the CNS survive to postnatal stages because the deletion is CNS-specific. It is unclear if the deletion of exons 11 and 12 of Talpid3 leads to the production of a protein lacking the region encoded by these exons. This could be verified only if we had access to an anti-Talpid3 antibody with greater specificity. Some of the mutations in TALPID3 found in the compound heterozygote JS patients are in exons that are transcript-specific and others are splice site mutations; therefore, it has been suggested that the mutant TALPID3 retains some of its function in JS patients, which may explain why there is no embryonic lethality [14]. Mutations in TALPID3 have also been found in lethal ciliopathies that are inherited in an autosomal recessive manner. In those cases, one of the mutations reported caused a nonsense-mediated decay of transcripts with exon 2, and the other the production of a transcript without exon 14 and an altered reading frame [51]. The severity of the phenotypes caused by mutations in TALPID3 thus appears to depend on the type of mutation and the transcript that is affected.

In summary, the hypoplastic cerebellum caused by reduced proliferation and survival of GCPs is one of the most striking developmental phenotypes of the Talpid3 mutant mice. This can be attributed to the loss of primary
cilia and compromised Shh signalling. Talpid3 has additional roles in cell migration and orientation. Deletion in Talpid3 affects cell migration, which disrupts the cellular organisation of the cerebellum. This in turn affects the cerebellar circuitry, which is the likely cause of ataxia. The Talpid3 conditional deletion mutant mouse recapitulates the hallmarks of Joubert syndrome. This, taken together with the identification of mutations in KIAA0586 (TALPID3) in some JS patients, establishes the Talpid3 CNS conditional mutant mouse as a model for JS.

Acknowledgements

We are very grateful to Professor Cheryll Tickle for providing us with the stock floxed Talpid3 mice and for her encouragement during the course of this investigation. The 2H3 monoclonal antibody developed by TM Jessell and J Dodd, HHMI, Columbia University, USA and the G3G4 monoclonal antibody developed by SJ Kaufman, University of Illinois, USA were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242, USA. We thank Jean Tye for assistance with the mouse colony. This research was funded by a University of Bath Alumni Funds grant to VS. ALB was supported by a Medical Research Council (UK) Doctoral training grant (MR/J500318).

Author contributions statement

ALB and VS designed the experiments and VS supervised the project. AB performed the experiments. ALB and VS analysed the data and wrote the manuscript.

References

1. Goetz SC, Anderson K. The primary cilium: a signalling centre during vertebrate development. Nat Rev Genet 2010; 11: 331–344.
2. Guemez-Gamboa A, Coufal NG, Gleeson JG. Primary cilia in the developing and mature brain. Neuron 2014; 82: 511–521.
3. Sattar S, Gleeson JG. The ciliopathies in neuronal development: a clinical approach to investigation of Joubert syndrome and Joubert syndrome-related disorders. Dev Med Child Neurol 2011; 53: 793–798.
4. Lee JE, Gleeson JG. Cilia in the nervous system: linking cilia function and neurodevelopmental disorders. Curr Opin Neurol 2011; 24: 98–105.
5. Sang LY, Miller JJ, Corbit KC, et al. Mapping the NPHP–JBS–MKS protein network reveals ciliopathy disease genes and pathways. Cell 2011; 145: 513–528.
6. Valente EM, Brancati F, Dallapiccola B. Genotypes and phenotypes of Joubert syndrome and related disorders. Eur J Med Genet 2008; 51: 1–23.
7. Maria BL, Quisling RG, Rosainz LC, et al. Molar tooth sign in Joubert syndrome: clinical, radiologic, and pathologic significance. J Child Neuro 1999; 14: 368–376.
8. Romani M, Micalizzi A, Valente EM. Joubert syndrome: congenital cerebellar ataxia with the molar tooth. Lancet Neurol 2013; 12: 894–905.
9. Valente EM, Silhavy JL, Brancati F, et al. Mutations in CEP290, which encodes a centrosomal protein, cause pleiotropic forms of Joubert syndrome. Nat Genet 2006; 38: 623–625.
10. Parisi MA, Doherty D, Chance PF, et al. Joubert syndrome (and related disorders) (OMIM 213300). Eur J Hum Genet 2007; 15: 511–521.
11. Louie CM, Gleeson JG. Genetic basis of Joubert syndrome and related disorders of cerebellar development. Hum Mol Genet 2005; 14: 235–242.
12. Stephen LA, Tawamie H, Davis GM, et al. Talpid3 controls centosome and cell polarity and the human ortholog KIAA0586 is mutated in Joubert syndrome (JBTS23). Elife 2015; 4: e08077.
13. Bachmann-Gagescu R, Phelps IG, Dempsey JC, et al. KIAA0586 is mutated in Joubert syndrome. Hum Mutat 2015; 36: 831–835.
14. Roosig S, Holfree M, Kim S, et al. Functional genome-wide siRNA screen identifies KIAA0586 as mutated in Joubert syndrome. Elife 2015; 4: e06602.
15. Malicdan MC, Vilboux T, Stephen J, et al. Mutations in human homologue of chicken talpid3 gene (KIAO586) cause a hybrid ciliopathy with overlapping features of Jeune and Joubert syndromes. J Med Genet 2015; 52: 830–839.
16. Kobayashi T, Kim S, Lin YC, et al. The CP110-interacting proteins Talpid3 and Cep290 play overlapping and distinct roles in cilia assembly. J Cell Biol 2014; 204: 215–229.
17. Davey MG, Paton IR, Yin Y, et al. The chicken talpid3 gene encodes a novel protein essential for Hedgehog signaling. Gene Dev 2006; 20: 1365–1377.
18. Yin YL, Bangs F, Paton IR, et al. The Talpid3 gene (KIAO586) encodes a centrosomal protein that is essential for primary cilia formation. Development 2009; 136: 655–664.
19. Bangs F, Antonio N, Thongmeye P, et al. Generation of mice with functional inactivation of talpid3, a gene first identified in chicken. Development 2011; 138: 3261–3272.
20. Ben J, Elworthy S, Ng AS, et al. Targeted mutation of the talpid3 gene in zebrafish reveals its conserved requirement for ciliogenesis and Hedgehog signalling across the vertebrates. Development 2011; 138: 4969–4978.
21. Sayer JA, Otto EA, O’Toole JF, et al. The centrosomal protein nephrocystin-6 is mutated in Joubert syndrome and activates transcription factor ATF4. Nat Genet 2006; 38: 674–681.
22. Baala L, Audollent S, Martinovic J, et al. Pleiotropic effects of CEP290 (NPHP6) mutations extend to Meckel syndrome. Am J Hum Genet 2007; 81: 170–179.
23. Leitch CC, Zaghloul NA, Davis EE, et al. Hypomorphic mutations in syndromic encephalocoele genes are associated with Bardet–Biedl syndrome. Nat Genet 2008; 40: 443–448.
24. Aguilar A, Meunier A, Strehl L, et al. Analysis of human samples reveals impaired SHH-dependent cerebellar development in Joubert syndrome/Meckel syndrome. Proc Natl Acad Sci U S A 2012; 109: 16951–16956.
25. Tronche F, Kellendonk C, Kretz O, et al. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat Genet 1999; 23: 99–103.
26. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. Nat Methods 2012; 9: 676–682.
27. Sillitoe RV, Joyner AL. Morphology, molecular codes, and circuitry produce the three-dimensional complexity of the cerebellum. Annu Rev Cell Dev Biol 2007; 23: 549–577.
28. Wang VY, Zoghbi HY. Genetic regulation of cerebellar development. Nat Rev Neurosci 2001; 2: 484–491.
SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Supplementary video legend

Figure S1. Growth and foliation of cerebella in Ta3 mutant and wild-type mice

Figure S2. Cell density and features of the EGL and IGL of the Ta3 mutant and wild-type cerebella

Figure S3. Primary cilia in Ta3 mutant and wild-type cerebella

Figure S4. Cell proliferation in E18.5 Ta3 mutant and wild-type cerebella

Figure S5. Apoptosis in the cerebella of Ta3 mutant and wild-type mice

Figure S6. Orientation of nuclei/cells in Ta3 mutant EGL

Figure S7. Bergmann glia are misplaced in the Ta3 mutant cerebellum

Figure S8. Morphology of the PCL, PCs, and dendritic arborisation in Talpid3 mutant and wild-type cerebella

Table S1. Primer sequences for qPCR

Video S1. Ta3 mutant mice exhibit severe ataxia but maintain grooming behaviour

© 2019 The Authors. The Journal of Pathology published by John Wiley & Sons Ltd on behalf of Pathological Society of Great Britain and Ireland. www.pathsoc.org

J Pathol 2019; 248: 396–408
www.thejournalofpathology.com