Expression patterns of ciliopathy genes ARL3 and CEP120 reveal roles in multisystem development

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Abstract

Background: Joubert syndrome and related disorders (JSRD) and Jeune syndrome are multisystem ciliopathy disorders with overlapping phenotypes. There are a growing number of genetic causes for these rare syndromes, including the recently described genes ARL3 and CEP120.

Methods: We sought to explore the developmental expression patterns of ARL3 and CEP120 in humans to gain additional understanding of these genetic conditions. We used an RNA in situ detection technique called RNAscope to characterise ARL3 and CEP120 expression patterns in human embryos and foetuses in collaboration with the MRC-Wellcome Trust Human Developmental Biology Resource.

Results: Both ARL3 and CEP120 are expressed in early human brain development, including the cerebellum and in the developing retina and kidney, consistent with the clinical phenotypes seen with pathogenic variants in these genes.

Conclusions: This study provides insights into the potential pathogenesis of JSRD by uncovering the spatial expression of two JSRD-causative genes during normal human development.

Keywords: CEP120, ARL3, Foetus, Development, Retina, Kidney, Brain, RNAscope

Background

Joubert syndrome and related disorders (JSRD) are a group of autosomal inherited ciliopathies that are characterised as a cerebello-retinal-renal phenotype, and have an incidence rate of 1:80,000–100,000 live births [1–3]. The hallmark brain phenotype is a “molar tooth sign” shown on axial brain MRI, caused by cerebellar vermis hypoplasia and other mid and hindbrain malformations [4]. These defects often cause symptoms of hypotonia, ataxia and intellectual disability in patients [5]. The retinal and renal phenotypes associated with JSRD have a lower incidence rate and vary in severity. Renal disorders occur in ~25% of patients, often presenting as corticomedullary cysts, interstitial fibrosis, or tubulointerstitial kidney disease [5]. The renal component is progressive and can lead to end-stage renal disease [6]. Ocular phenotypes of retinitis dystrophy, retinitis pigmentosa, oculomotor apraxia, and ptosis are common in patients, and as with the renal aspects of JSRD are often progressive in nature [7].

Currently, there are more than 35 genes that are known to cause JSRD (https://www.omim.org/phenotypicSeries/PS213300). The syndrome is caused by defects...
of the primary cilia, which are found on most mammalian cells [8]. Primary cilia act as a cellular antenna to transduce extracellular signals such as mechanical flow, chemical stimulation, and key signalling pathways (including Hedgehog, Wnt, and PDGF) into the cell [9–13]. Due to the multi-organ involvement, varying phenotypes, and multitude of genes known to cause JSRD there is great heterogeneity within the syndrome and overlap with closely related ciliopathies including Bardet-Biedl syndrome (https://omim.org/phenotypic-Series/PS209900) and Jeune syndrome (https://omim.org/phenotypicSeries/PS208500) [14]. Recently discovered genetic causes of JSRD include ARl3 [15] and CEP120 [16, 17]; the fact that their encoded proteins have such divergent roles within the primary cilium demonstrates the complexity underlying this group of related disorders.

ADP-ribosylation factor-like 3 (ARl3), a RAS superfamily member, is a low molecular weight GTP-binding protein [18] that cycles between inactive GDP-bound and active GTP-bound states to release cargo from their carriers in the cilium [19]. ARl3 interacts with its Guanine Exchange Factor ARl3B in the cilium [20] and GTPase Activating Protein RP2 at the basal body of the cell [21, 22]. Arl3 knockout studies in mice demonstrate a multi-organ ciliopathy phenotype, including kidney cysts, liver fibrosis and retinal disease with photoreceptor cell degeneration [23–26]. Recently, two families affected by JSRD have been identified, presenting with ciliopathy phenotypes [15]. The underlying genetic cause was shown to be missense mutations in ARl3, which affect an amino acid residue involved in the interaction between ARl3 and ARl3B [15].

Centrosomal protein of 120 kDa (CEP120) is a centrosomal protein involved in centriole biogenesis, including centriole duplication, assembly [27, 28], elongation [29–31] and maturation [28]. CEP120 also interacts with other centrosomal proteins including CPAP [29, 30], SPICE1 [29], Talpid3 [28, 31] and C2CD3 [31]. CEP120 was found to be expressed ubiquitously in murine embryonic tissues such as the brain, kidney and lungs. Additionally, Cep120 was observed to be highly expressed in embryonic mouse brain compared to postnatal or adult mouse brain [32]. Inactivation of CEP120 in the mouse central nervous system results in hydrocephalus and cerebellar hypoplasia [28]. CEP120 mutations have been shown to cause JSRD and Jeune syndromes [16, 17], and overlapping ciliopathy phenotypes such as tectocerebellar dysraphia with occipital encephalocese (TCDOE), Meckel syndrome (MKS) and oro-facial-digital (OFD) syndromes [17].

The developmental expression patterns of ARl3 and CEP120 in humans is not known. In order to explore this, we used an RNA in situ detection technique called RNAscope to compare and contrast the developmental spatial expression of these new and divergent causes of JSRD. We successfully characterised ARl3 and CEP120 expression patterns in human embryos and foetuses in collaboration with the MRC-Wellcome Trust Human Developmental Biology Resource (HDBR). This study provides insights into the potential pathogenesis of JSRD by uncovering the expression pattern of two JSRD-causative genes during normal human development.

Methods

RNAscope studies

Characterisation of ARl3 and CEP120 expression patterns was performed in human embryonic tissue using samples obtained from the MRC-Wellcome HDBR. Formalin fixed paraffin embedded sections of human embryonic and foetal tissue were prepared using 10% neutral buffered formalin and fixed for 32 h at room temperature. Samples were then prepared for the RNAscope assay, a RNA in situ detection platform for detection of target RNA within intact cells, as per manufacturers’ instructions [33, 34]. An RNAscope 2.5 Assay RED was employed with 20 paired probes across nucleotides 169–1570 (NM_004311.3) and 115–1133 (NM_001166226.1) for detection of ARl3 and CEP120, respectively and counterstained with Methyl Green.

Whole human embryo sections of 8 post-conception weeks (PCW), (equivalent to Carnegie Stage 23) were analysed, along with hindbrain (14PCW and 19PCW), eye (14PCW), kidney and adrenal gland (14PCW and 18PCW). A negative RNAscope 2.5 HD Assay Red control (dapB, a bacterial gene which is not expressed in human tissues) was performed (Supplementary Figure 1). In addition, the RNAscope 2.5 HD Assay RED was performed with a positive control (Ki67, a cell proliferation marker). Ki67 is a nuclear protein commonly used as a proliferation marker, which is expressed in cycling cells and is associated with cellular proliferation. It is encoded by the gene marker of proliferation Ki-67, MKI67. ARl3 and CEP120 human expression patterns were analysed using the HDBR image server (Leica Biosystems).

Clinical phenotypes and sequence analysis

Reported clinical phenotypes associated with ARl3 and CEP120 mutations were reviewed within OMIM (https://omim.org/). Putative Arl3 and Cep120 orthologues were identified using BLASTP, with human ARl3 (isoform a, NP_004302.1) and CEP120 (NP_694955.2) transcripts as the query sequences within NCBI (https://www.ncbi.nlm.nih.gov/). Additional databases including Flybase (https://flybase.org/), Wormbase (https://www.wormbase.org/) and Phytozome (https://phytozome.jgi.doe.gov/) were also queried using BLAST.
Results

Clinical phenotypes of ARL3 and CEP120 patients

Biallelic mutations in both ARL3 and CEP120 mutations are rare causes of ciliopathy syndromes. A comparison of the known phenotypes associated with ARL3 and CEP120 mutations is shown in Table 1. This overview reveals that mutations in CEP120 are at present associated with severe phenotypes including MKS but also that single heterozygous changes in ARL3 are sufficient to cause retinal-limited phenotypes. ARL3 is highly conserved, with homologs present in C. elegans, C. reinhardtii and D. melanogaster whereas CEP120 appears not to have homologs within these lower organisms (Supplementary Table 1). Known protein localisation within the cell of both ARL3 within the ciliary axoneme, and CEP120 in the centrosomes, are consistent with their role in ciliopathy syndromes (Supplementary Table 2).

ARL3 and CEP120 are expressed in early human brain development

In 8PCW human brain tissue, the expression of ARL3 and CEP120 is remarkably similar. There is expression of both genes in the choroid plexus (Fig. 1Ai and 1Bi), which appears to favour the luminal facing surface of the tissue, especially for ARL3. The cell proliferation marker Ki67 does not share this same expression pattern in the choroid plexus (Fig. 1Ci). This specific expression pattern of ARL3 and CEP120 in luminal-facing cells is continued throughout the developing brain where both genes exhibit expression throughout the ventricular zone of the ganglionic eminences, cortical wall, and the hind-brain including the rhombic lip (Fig. 1Aii-Aiv and 1Bii-Biv). There is specific expression of ARL3 and CEP120 in the layer of cells forming the apical surface in each tissue, facing into the ventricular space. Expression of Ki67 is seen throughout these tissues (Fig. 1Cii-Civ), with specific expression in the apical layer consistent with this being the major site of cell division (cells in G2/M1 phase of cell cycle) in the ventricular zone [37].

Expression of ARL3 and CEP120 is maintained in the developing cerebellum

In the human cerebellum at 14PCW there is expression of ARL3 and CEP120. Both genes have strong expression in the external and internal granule cell layer (EGL and IGL) the developing cerebellum (Fig. 2Ai and Ci). Expression in the EGL and IGL is seen at 19PCW for ARL3 (Fig 2Bi) however, CEP120 expression is predominantly localised in the EGL and the molecular layer (ML) of the cerebellum at 19PCW (Fig. 2Di). Strong expression of Ki67 is seen throughout the EGL in particular, but also the IGL at 19PCW, indicating the tissue is proliferative (Fig. 2Ei). ARL3 and CEP120 are therefore widely expressed in the cerebellum during development, with specific expression of CEP120 in the ML which is

Table 1 Comparison of the known phenotypes associated with ARL3 and CEP120 mutations

| Patients with ARL3 related ciliopathy | Patients with CEP120 related ciliopathy |
|--------------------------------------|---------------------------------------|
| **Number of affected patients reported and presenting phenotypes** |
| 4 patients reported with JSRD secondary to biallelic changes [15] |
| 4 patients with retinitis pigmentosa secondary to monoallelic changes [35, 36] |
| **Brain imaging findings** |
| Molar tooth sign |
| **Intelect** |
| Developmental delay |
| Psychomotor delay |
| **Skeletal** |
| No known associated phenotypes |
| **Mobility** |
| Ataxic gait |
| Hypotonia |
| **Eye** |
| Night blindness |
| Bilateral vision loss |
| Retinal dystrophy |
| Ocular motor apraxia |
| **Kidney** |
| Cystic dysplastic kidney |
| Bilateral renal scarring |
| Recurrent urinary tract infections |

*JSRD* Joubert syndrome and related disorders; *JATD* Jeune asphyxiating thoracic dystrophy; *MKS* Meckel syndrome; *TCDOE* tectocerebellar dysraphia with occipital encephalocele
predominantly occupied by the dendritic trees of Purkinje cells and the interacting parallel fibres of granule cells. As dendrites and axons contain low levels of mRNA, it is likely that CEP120 expression is predominantly located in the sparse population of interneurons found in the molecular layer [38] or in immature granule cells migrating from EGL to IGL [39] suggesting a role for CEP120 in these cell types [38].

Expression of ARL3 and CEP120 in the developing eye
The human retina can be divided into nine layers based upon the cell types that occupy each layer

Fig. 1 Expression pattern of ARL3 and CEP120 in the human brain during early development. Sagittal sections of 8PCW-stage human embryos stained using RNAscope to show expression of ARL3 (a) (red), CEP120 (b) (red) and KI67 (c) (red), counterstained with Methyl Green. Aii and Bi
ARL3 and CEP120 are expressed within cells of the choroid plexus (arrow). (C) KI67 expression is minimal in the choroid plexus. Aii-Aiv and (Bii-Biv) Expression of ARL3 and CEP120 is seen in the ventricular radial glia progenitor cells including the ventricular zone of the ganglionic eminences (Aii and Bii), cerebral cortex (Aiii and Biii), and rhombic lip (Aiv and Biv). Cii-Civ Expression of KI67 is seen in the ventricular zone of the ganglionic eminences, cerebral cortex, and hindbrain
(Fig. 3a), with the retinal pigment epithelial (RPE) and photoreceptor layers at the outermost part of the eye [39]. At 8PCW, the retinal layers are not well defined with only a ganglion cell layer separated from a layer of mostly immature neuroblasts with a few photoreceptor cells by a thin inner plexiform layer [40]. At this stage ARL3 and CEP120 show expression throughout the developing retina, with high expression within the retinal ganglion cells and the photoreceptor layer (Fig. 3Bi and Di). At 14PCW, the retinal layers are maturing [39] which is reflected in the expression pattern of both ARL3 and CEP120. Clear expression of both genes is still seen in all layers of the retina, although to a lesser extent in the plexiform and nerve fibre layers due to reduced cell density in these areas (Fig. 3Ci and Ei).

**Expression of ARL3 and CEP120 in the developing dorsal root ganglia**

The dorsal root ganglia are formed by migrating neural crest cells and contain most of the body’s sensory neurons [42, 43]. Both ARL3 and CEP120 show expression in cells of the dorsal root ganglia, which are post-mitotic primary sensory neurons (Fig. 4 Ai-Aii and Bi-Bii). There is strong expression of Ki67 within limited
Fig. 3 Expression of ARL3 and CEP120 in the developing human retina. a Schematic diagram of the development of the layers of the retina from 8PCW to the mature form (adapted from [40]). At 8PCW, not all of the layers are present in the retina. The ONbL is a transitionary layer containing retinal progenitor cells that will develop into various cell types such as photoreceptors, amacrine and bipolar cells; separating into the ONL, OPL, INL, and IPL (the IPL is sometimes visible at 8PCW). The GCL is thicker at 8PCW due to the migration of cells. The mature retina can be divided into layers. The RPE is at the very back of the eye and assists in the removal of waste products from the photoreceptor cells, which transduce light. The ONL, OPL, INL and IPL layers house intermediary cell bodies and dendrites that interact with ganglion cells in the GCL to convey the signal through the optic nerve, formed in the NFL, to the brain (reviewed in [41]). b Human sections of developing eye at 8PCW (b and d) and 14PCW (c and e) stained using RNA Scope to show ARL3 expression (b and c) (red) and CEP120 (d and e) (red) counterstained with Methyl Green (Bi and Di). There is a gradient of ARL3 (Bi) and CEP120 (Di) expression in the retina at 8PCW across multiple retinal layers including the ONbL. At 14PCW, ARL3 (Ci) and CEP120 (Ei) expression is localised across multiple layers including the photoreceptor cell layer, just below the RPE layer (arrows). GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NFL, nerve fibre layer; ONL, outer nuclear layer; OPL, outer plexiform layer; ONbL, outer neuroblastic layer; RPE, retinal pigment epithelium.
number of cells in the dorsal root ganglia, presumably non-neuronal (Fig. 4 Ci-Cii).

Expression of ARL3 and CEP120 in the developing kidney
In the developing human kidney at 8PCW, where there is strong renal cortical staining of KI67 indicating cell proliferation (Supplementary Figure 2), there is expression of ARL3 in cells within the developing cortical nephrons; this expression appears to be specifically oriented to the lumen of the structures (Fig. 5Ai). This expression pattern is maintained at 14PCW (Fig. 5Bi) and 18PCW (Fig. 5Ci). Expression of CEP120 is also seen in developing nephrons at 8PCW, however there is also expression in the renal cortex (Fig. 5Di). This expression pattern of CEP120 is maintained at 14PCW (Fig. 5Ei) and 18PCW, although overall expression appears to have decreased at this time point (Fig. 5Fi).

Expression of ARL3 and CEP120 in other major organs
In the developing human heart, lung and gut at 8PCW, there is very low levels of expression of ARL3 and CEP120 (Supplementary Figure 3). Expression of ARL3 and CEP120 is seen around the developing alveoli and at low levels in the developing bowel epithelia. The remaining organs of the developing embryo did not reveal prominent expression patterns.

Discussion
Mutations in ARL3 and CEP120 are rare and relatively new causes of JSRD and other related ciliopathies. Human protein atlas data suggests that tissue expression of ARL3 protein is widely expressed, with highest expression scores seen in cerebellum and lowest in heart and skeletal muscle (https://www.proteinatlas.org/ENSG00000138175-ARL3/tissue). RNA expression is high in cerebral cortex, cerebellum, retina and kidney consistent with its known phenotypes. CEP120 protein expression is not annotated within the human protein atlas, whereas RNA is strongly expressed in the cerebellum (https://www.proteinatlas.org/ENSG00000168944-CEP120/tissue). We aimed to define expression of ARL3 and CEP120 during human development using the HDBR tissue bank employing a relatively new in situ hybridisation assay called RNAscope for the detection of target RNA within intact cells. We used KI67 as a positive control although we recognise that expression of KI67 is not homogeneous throughout each tissue. Our data provide an insight into the developmental expression of ARL3 and CEP120. We show that both of these genes are expressed in key tissues (including retina, cerebellum and kidney) during development. This expression pattern fits with the multisystem disease phenotypes seen in patients with ARL3 and CEP120 mutations (Table 1). A similar approach, using the valuable HDBR tissue bank has been performed, using in situ hybridisation for studying the expression of ARL13B [44], another cause of Joubert syndrome. Here ARL13B was detected at stage CS16 in the alar and basal plate of the myelencephalon, the mesencephalon and the metencephalon. At CS19 ARL13B was seen in the ventricular layer of the diencephalon and myelencephalon, the tegmentum of the pons and the cerebellar rhombic lips as well as the dorsal root ganglia. This pattern of expression is remarkably similar to the CEP120 and ARL3 data described here.

Expression of both ARL3 and CEP120 was minimal in developing cardiac, lung and gut tissues, consistent with lack of known phenotypes affecting these organ systems (Supplementary Figure 3). ARL3 and CEP120 encode proteins that are expressed in the primary cilia and basal body respectively (Supplementary Table 2) and pathogenic variants result in similar and overlapping phenotypes, including the cerebello-retinal-renal syndrome JSRD (Table 1). The number of patients with pathogenic variants in either ARL3 or CEP120 remains small, allowing a limited comparison of phenotypes, although skeletal manifestations (in particular short ribs/asphyxiating thoracic dystrophy phenotypes) seen in patients with CEP120 mutations have not been documented in patients with ARL3 mutations.

There were notable differences in evolutionary conservation between ARL3 and CEP120 (Supplementary Table 1). The ARL3 human protein shares greater than 90% identity with its two orthologous sequences (there is genomic duplication of arl3) in Danio rerio (zebrafish), a well-studied model species in vertebrates. In contrast, CEP120 human protein only shares 57% identity with its single orthologous sequence found in zebrafish. Moreover, human ARL3 protein shares > 60% identity with its orthologues found in Drosophila melanogaster, Caenorhabditis elegans and Chlamydomonas reinhardtii. CEP120 is conserved in some vertebrate organisms but orthologues were not readily identified in invertebrates. There is a putative CEP120 orthologue, UNI2, found in Chlamydomonas reinhardtii, but this has not as yet been confirmed as a functional ortholog [27, 44]. ARL3 is described in diverse eukaryotic organisms such as Leishmania donovani [45] and Caenorhabditis elegans [46] [47] where it has a functional role in the cilium/flagella. Despite these differences in evolutionary conservation, our results show that ARL3 and CEP120 have similar expression patterns during human development, specifically in the eye and dorsal root ganglia as well as during early brain development. Both genes are expressed throughout the retina during development, with expression in the RPE and photoreceptor layers, suggesting a role for both genes during retinal development. This is further supported by the numerous retinal phenotypes associated with mutations in ARL3 [15–17]. Similarly, the specific expression of ARL3 and CEP120 in
the dorsal root ganglia hints at a role for both genes in primary sensory neurone differentiation. A recurring pattern was the expression of both mRNAs on the luminal facing surface of the cerebral tissue (seen in the choroid plexus and ventricular zones of the cerebral cortex ganglionic eminences, and hindbrain) which could suggest a sensory role for the gene products of both the genes within the cilium of the ventricular lining of the brain.

Expression of \textit{ARL3} and \textit{CEP120} changes during development notably in the cerebellum and kidney. \textit{ARL3} and \textit{CEP120} are expressed throughout the cerebellum at 14PCW however, at 19PCW, \textit{ARL3} expression was predominantly in the IGL whereas \textit{CEP120} was expressed in the EGL and ML of the cerebellum. This could imply that \textit{ARL3} and \textit{CEP120} are expressed in different cell populations of the cerebellum, \textit{ARL3} in both immature and mature granule cells, and \textit{CEP120} in immature, migratory granule cells and ML interneurones [48]. It has been previously reported in mouse studies that \textit{Cep120} is required for proliferation of cerebellar neural progenitor cells [28] and is required for correct development of the embryo. Taken with these results, it suggests that \textit{CEP120} expression is required for correct development of the cerebellum in humans.

Expression of \textit{ARL3} and \textit{CEP120} also differed in the developing kidney. The results showed that \textit{ARL3} was specifically expressed in cells of the nephrons whereas \textit{CEP120} was expressed in the nephrons as well as within cells in the developing renal cortex. This difference in expression could imply that \textit{ARL3} has a more sensory/signalling function in luminal structures of the kidney, whereas \textit{CEP120} has a more universal role in all cells as it is expressed more ubiquitously throughout the tissue.

The differences in gene expression may reflect the divergent functions of \textit{ARL3} and \textit{CEP120} proteins (Supplementary Table 2). As \textit{ARL3} is a trafficking protein involved in ciliary signalling [19, 49], it may only be expressed in actively signalling cells during certain points in development such as nephron progenitors and cells in the IGCL. In contrast, \textit{CEP120} is involved in building the centriole, and therefore cilium, [27, 29] and so will be expressed more widely within tissues, especially those with ciliated epithelia [50, 51].
In conclusion, we establish in human embryonic tissue expression patterns of ARL3 and CEP120 during development and provide insights into the wide phenotypic spectrum of mutations affecting ARL3 and CEP120 in humans. These studies will allow further investigations into tissue-specific mechanistic roles of ARL3 and CEP120 in human health and disease.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12861-020-00231-3.

Additional file 1.

Abbreviations
EGL: External granule cell layer; GCL: Ganglion cell layer; HDBR: Human Developmental Biology Resource; IGL: Internal granule cell layer; INL: Inner nuclear layer; IPL: Inner plexiform layer; JATD: Jeune asphyxiating thoracic dystrophy; JSRD: Joubert syndrome and related disorders; MKS: Meckel syndrome; ML: Molecular layer; NFL: Nerve fibre layer; OFD: Oro-facial-digital; ONbL: Outer neuroblastic layer; ONL: Outer nuclear layer; OPL: Outer plexiform layer; PCW: Post-conception weeks; RPE: Retinal pigment epithelium; TCDOE: Tectocerebellar dysraphia with occipital encephalocele

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Authors’ contributions
LP and MB-G analysed and interpreted the data regarding the RNA expression studies. GJC, LAD, SAR and CGM performed data analysis and contributed in writing the manuscript. JAS conceived the project. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
This study was conducted with full ethical approval. For human embryonic and foetal tissue samples, the samples were collected with appropriate written maternal consents and ethical approval by the Newcastle and North Tyneside 1 Research Ethics Committee, UK.

Consent for publication
Not applicable.
Competing interests
The authors declare that they have no competing interests.

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