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SPECC1L: a cytoskeletal protein that regulates embryonic tissue dynamics

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Abstract

Many structural birth defects occur due to failure of tissue movement and fusion events during embryogenesis. Examples of such birth defects include failure of closure of the neural tube, palate, and ventral body wall. Actomyosin forces play a pivotal role in these closure processes, making proteins that regulate actomyosin dynamics a priority when studying the etiology of structural birth defects. SPECC1L (sperm antigen with calponin homology and coiled-coil domains 1 like) cytoskeletal protein associates with microtubules, filamentous actin, non-muscle myosin II, as well as membrane-associated components of adherens junctions. Patients with *SPECC1L* mutations show a range of structural birth defects affecting craniofacial development (hypertelorism, cleft palate), ventral body wall (omphalocele), and internal organs (diaphragmatic hernia, bicornuate uterus). Characterization of mouse models indicates that these syndromic mutations utilize a gain-of-function mechanism to affect intra- and supra-cellular actin organization. Interestingly, SPECC1L deficiency appears to affect the efficiency of tissue dynamics, making it an important cytoskeletal regulator to study tissue movement and fusion events during embryonic development. Here we summarize the *SPECC1L*-related syndrome mutations, phenotypes of *Specc1l* mouse models, and cellular functions of SPECC1L that highlight how it may regulate embryonic tissue dynamics.

Keywords

scaffolding protein; actin; microtubules; palatogenesis; birth defects

Introduction

Embryonic development involves many instances of tissue movement that culminate in adhesion and fusion. Improper or incomplete movement or fusion of these tissues leads to common structural birth defects. Examples of such instances in development include

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closure of the neural tube, palatal shelves, ventral body wall, optic cup, and diaphragm; failure of these processes lead to birth defects of exencephaly (or spina bifida), cleft palate, omphalocele, coloboma, and diaphragmatic hernia, respectively (1, 2). While each individual event is complex and involves different cell types and movement events, there may be a common role for actomyosin forces (3, 4). In order for these actomyosin forces to participate in tissue movement, the actin filaments (F-actin) and non-muscle myosin II (NMII) need to be organized at both the intracellular and supracellular levels. Many cytoskeletal proteins that play a role in such organization of F-actin and NMII have been identified (5). Sperm antigen with calponin homology and coiled-coil domain 1 like (SPECC1L) is another actomyosin regulator with one important distinction – SPECC1L is not necessary for the initiation or even completion of these tissue movement and fusion events, but rather SPECC1L appears to modulate the efficiency or dynamics of the process.

In a cell, SPECC1L is found to associate with F-actin (6, 7), microtubules (6–8) and non-muscle myosin II (NMII) (6). In addition, SPECC1L expression concentrates at cell boundaries, particularly in epithelial cells at high density or in tissue, likely associated with adherens junction markers (9, 10). Expression is also observed in pericentriolar region and, in a dividing cell, in the mitotic spindle (8). Thus, SPECC1L appears to function as a cytoskeletal scaffolding protein. SPECC1L protein contains several coiled-coil domains (CCDs) – depending on the stringency of the prediction criteria, there are at least 3 CCDs (Fig.1) (7, 8, 10). CCD1 can be further split into two, and CCD3 into five smaller CCDs (Fig.1). While the role of CCD1 and CCD3 is not yet clear, several studies have confirmed that CCD2 is critical for association with microtubules (6–8, 10, 11). In addition to CCDs, SPECC1L also contains a calponin homology domain (CHD), known to bind F-actin. Most of the work thus far has been performed on CCD2 and CHD due to their significance in human disease. Human autosomal dominant mutations that largely cluster in CCD2 and CHD result in similar consequences (Fig.1), both at the level of human phenotypic manifestation (12, 13), and in the cell where they result in poor association with microtubules (8, 10, 11). Thus, we will begin with summarizing human *SPECC1L* mutations and accompanying defects, then present data from mouse models that support these mutations as being gain-of-function, and lastly present cellular and molecular evidence indicating SPECC1L likely functions to regulate actomyosin forces underlying tissue movement and fusion dynamics.

Phenotypic profile of patients with *SPECC1L* mutations

SPECC1L-related syndrome mutations

SPECC1L mutations were first reported in two patients – the first with bilateral Tessier IV type oblique facial clefts, cleft palate, severe bilateral ocular hypoplasia and clubfoot, and the second with unilateral Tessier IV cleft, contralateral Tessier VII lateral cleft, cleft palate and unilateral ocular hypoplasia (8). The first patient had a balanced chromosomal translocation resulting in apparent *SPECC1L* haploinsufficiency, while the other patient had a Q415P mutation affecting the CCD2 domain. Autosomal dominant *SPECC1L* mutations were later reported in several patients originally identified with either autosomal Opitz G/BBB or Teebi hypertelorism syndrome (11, 12, 14). The phenotypic similarity

among these patients has led to this being characterized as *SPECC1L*-related syndrome (13) or *SPECC1L*-related hypertelorism syndrome (Orphanet, #ORPHA:1519). The main structural anomalies included hypertelorism, cleft palate, omphalocele, ventriculomegaly, diaphragmatic hernia and bicornuate uterus (Fig.2). In addition, several patients suffered with developmental delay, intellectual disability or behavioral deficits including autism. While several of the structural defects involve tissue movement and fusion, not all do. Due to the range of systems affected, it is important to note that SPECC1L has a broad role in embryonic development beyond tissue movement and fusion.

The most striking aspect of *SPECC1L*-related syndrome autosomal dominant mutations is that they largely cluster in CCD2 and CHD (Fig.1), indicating a significant role for both SPECC1L-microtubule and SPECC1L-actin interactions.

Nonsyndromic CL/P mutations

Hall *et al.* (10) identified a few functional *SPECC1L* variants in nonsyndromic cleft lip and/or palate (nsCL/P) patients (Fig.1). While CL/P is associated with numerous well-characterized syndromes (ex. *SPECC1L*-related syndrome), most instances of CL/P are not associated with a syndrome and manifest as isolated or nsCL/P. The genetic etiology of nsCL/P is complex with both genetic and environmental influences. Variants in many syndromic CL/P genes have been identified in nsCL/P patients. The consensus in the field is that these nsCL/P associated variants have subtle functional consequences compared to syndromic variants in the same gene, and that nsCL/P variants in multiple genes combine to reach the genetic threshold sufficient to manifest this complex disease (15–18). Consistent with this general opinion, Hall *et al.* (10) found that nsCL/P associated *SPECC1L* variants were outside of CCD2 or CHD, and showed milder functional consequences than *SPECC1L* syndromic variants.

Loss-of-function mutations?

According to gnomAD (19, 20), a large database containing exome and genomic sequences of >140,000 individuals from the general population, there is a significantly lower occurrence of heterozygous *SPECC1L* loss-of-function alleles than expected. The gnomAD LOEUF (loss-of-function observed/expected upper bound fraction) score is a conservative estimate of the observed/expected ratio, based on the upper bound of a Poisson-derived confidence interval around the ratio. *SPECC1L* LOEUF score is a 0.33 on a scale of 0–1. Low LOEUF scores indicate strong selection against predicted loss-of-function (pLoF) variation in a given gene, while high LOEUF scores suggest a relatively higher tolerance to inactivation. A low LOEUF score indicates a strong selection against predicted loss-of-function variants, implying that heterozygosity of the gene is not tolerated well during development and that the gene may be associated with severe disease or mortality. Consistently, there is no evidence of homozygous loss of *SPECC1L* in any database, likely because it is embryonic lethal based on our mouse data summarized below. This also suggests that *SPECC1L* heterozygosity likely leads to a different phenotypic manifestation than that for CCD2/CHD based *SPECC1L*-related syndrome. However, no *SPECC1L* loss-of-function mutations, associated with any embryonic deficits or lethality, have yet been reported in the literature.

Mouse models of *Specc1l* deficiency

Null and truncation alleles

Several *Specc1l* mouse alleles with truncations or overall deficiency have been generated (6, 9, 10). In general, homozygous mutants for null and truncation alleles showed perinatal lethality (6, 10), indicating that SPECC1L function is needed for survival. These null homozygous mutant embryos in general show midgestational edema, which in severe cases would extend medially from the cranium down along the length of the spine. In addition, these mutants showed short stature, abnormal craniofacial features and syndactyly of digits 2/3 in both forelimbs and hindlimbs (21). Further characterization showed palatogenesis defects in these null mutants, including a delay in palatal shelf elevation as well as delayed and incomplete palatal rugae formation (10). Prevalence of the delayed palatal shelf elevation phenotype was ~67% among homozygous null mutant embryos on the C57BL/6J background. Yet, remarkably, none showed cleft palate at birth (6, 10). Hall *et al.* (10) proposed that this delay in palatal shelf elevation makes these mutants susceptible to cleft palate, and asserted that *SPECC1L* variants identified in nsCL/P patients may serve to increase susceptibility to CL/P.

CCD2 in-frame deletion alleles

The *Specc1l* null allele was perinatal lethal and showed palatal shelf elevation delay, but did not show cleft palate phenotype at birth (6). In contrast, homozygous mutants for mouse alleles with short in-frame deletions removing portions of the CCD2 (collectively termed *ΔCCD2*) showed ~50% exencephaly (open cranial neural folds) and ~50% cleft palate at birth (6). Importantly, 10–20% of *ΔCCD2* heterozygotes, depending on background, showed cleft palate, consistent with the autosomal dominant manifestation human patients with *SPECC1L* mutations. Interestingly, none of the *ΔCCD2* exencephalic mutants showed cleft palate. A more detailed analysis showed a severe palatal shelf elevation delay in all *ΔCCD2* homozygous mutants, however, the oral cavity was found to be narrower in mutants with exencephaly. Thus, Goering *et al.* (6) argues that the narrow oral cavity in exencephalic mutants allowed palatal shelves to elevate and close despite the delay. In addition to the neural tube and palatal shelves, Goering *et al.* (6) also observed failure in closure of the ventral body wall (omphalocele), optic cup (coloboma), and eyelids at birth. These results indicated that loss of SPECC1L-CCD2 function impacted the efficiency of movement and fusion of multiple tissues. Importantly, in addition to cleft palate, the omphalocele phenotype occurred in ~48% of patients with *SPECC1L*-related syndrome (Fig.2) (12). Thus, recapitulation of the patient phenotypes in the *ΔCCD2* mutant alleles, but not the null alleles, indicated a gain-of-function mechanism for syndromic CCD2 (and CHD) mutations.

Taken together, the difference in phenotypes between null and *ΔCCD2* mouse mutants further suggest that, when identified, *SPECC1L* loss-of-function variants in humans will likely manifest in a different phenotypic spectrum than that from CCD2/CHD point mutations.

Cellular function of SPECC1L

Association with microtubules required for cell trafficking

In a normal cell, there is some overlap of SPECC1L expression with that of microtubule markers such as beta-tubulin (6). As stated above, SPECC1L associates strongly with the mitotic spindle, which consists of acetylated microtubules. Further, when a SPECC1L-GFP construct was overexpressed ectopically, it associated with a subset of microtubules that were also acetylated (8, 10). This ectopic association was diminished with the introduction of *SPECC1L*-related syndrome CCD2 mutations (8–11). Thus, SPECC1L association with microtubules likely requires CCD2. Interestingly, overexpression of the subtle nonsyndromic CL/P variants identified outside of CCD2 or CHD, showed no change in association with microtubules but the acetylation of SPECC1L-bound microtubules was delayed (10), suggesting that SPECC1L binds to a subset of microtubules and facilitates acetylation. It remains to be seen whether SPECC1L functions similarly *in vivo* in acetylation of microtubules in the cytoplasm or that form the mitotic spindle.

In primary mouse embryonic palate mesenchymal (MEPM) cells (22–24) from *ΔCCD2* mutants, the SPECC1L-ΔCCD2 protein did not associate with microtubules and accumulated in regions around the nucleus (6). In these *ΔCCD2* mutant cells, the microtubule network itself did not appear to be altered, thus indicating that CCD2-based microtubule association is mainly required for intracellular trafficking of SPECC1L (Fig.3) (6). It remains possible that other regions of SPECC1L may facilitate other SPECC1L-microtubule functional interactions.

Association with actin filaments and their turnover

As mentioned previously, the most prominent expression of endogenous SPECC1L in the cell is observed overlapping that of F-actin (6, 7), likely due to presence of the C-terminal CHD, which is known to facilitate association with F-actin (25, 26). Recent studies by Mehta *et al.* (7) show presence of two WASP homology domain-2 (WH2) motifs (Fig.1), which facilitate binding to monomeric actin (27, 28). The significance of SPECC1L association with monomeric actin is not yet understood. Importantly, the most prominent cellular defect upon SPECC1L deficiency is in the organization of F-actin (6, 8, 9). In *Specc1l* homozygous null mutant MEPM and knockdown cells, F-actin staining is increased indicating that SPECC1L may participate in F-actin turnover. In homozygous *ΔCCD2* mutant MEPM cells, the region around the nucleus where the SPECC1L-*Δ*CCD2 mutant protein clusters showed a drastic decrease in F-actin staining. In contrast, at the cell periphery of these *ΔCCD2* mutant cells, there was a significant increase in F-actin staining (6). Thus, while CCD2 is required for subcellular localization of SPECC1L, it does not appear to be required for the F-actin turnover function of SPECC1L (Fig.3). How SPECC1L facilitates F-actin turnover is not yet known. The best-known mechanism of F-actin turnover is via actin depolymerizing factor cofilin (ADF/Cofilin) (29, 30). Since SPECC1L-associated actin filaments are generally thinner and F-actin turnover occurs at the site of SPECC1L cellular localization, determining whether SPECC1L recruits ADF/Cofilin will be of interest.

While the mechanism of SPECC1L-based F-actin turnover is not yet clear, the current evidence strongly indicates that the extent of actin cytoskeletal disorganization upon SPECC1L deficiency correlates with the severity of tissue movement and fusion defects. The *Specc1l-ΔCCD2* mutant cells show a much more disorganized actin cytoskeleton than the *Specc1l*-null mutant cells (6). Consistently, the *ΔCCD2* mutant embryos show multiple fusion defects at birth (exencephaly, cleft palate, omphalocele, coloboma), while the null mutants show some delay but no tissue movement and fusion defects at birth (6).

Association with non-muscle myosin II and myosin phosphatase complex

SPECC1L was shown to associate with both non-muscle myosin IIA (NMIIA, MYH9) and NMIIB (MYH10) in co-immunoprecipitation assays (6). This interaction appears independent of CCD2 or CHD, implying that there is a separate NMII interaction domain in SPECC1L. Further, this interaction implicates SPECC1L in the regulation of actomyosin forces. Both neural tube and ventral body wall closure have been shown to involve actomyosin-based contraction (31–34). While actomyosin contractility has not been directly implicated in palate closure, mouse mutants for a point mutation in *Myh10* resulted in cleft palate, omphalocele and diaphragmatic hernia (35). Further, single nucleotide polymorphisms in *MYH9* have been associated with nsCL/P in humans (36, 37). The NMII organization also showed more deterioration in *ΔCCD2* mutant cells than in *Specc1l*-null cells (6). There remains a possibility that NMII organization defects are secondary to those of F-actin turnover. Specific deletion of SPECC1L NMII binding domain is required to dissect out the significance of SPECC1L-NMII interaction.

The importance of SPECC1L in actomyosin dynamics is further illustrated by the recent discovery of its direct association with the myosin phosphatase complex MYPT1/PP1β (7). Through its role in dephosphorylation of the myosin light chain, this complex is an important regulator of actomyosin dynamics in both muscle and non-muscle cells. SPECC1L, which is known to associate with both microtubules and F-actin, may play an important role in the distribution of the myosin phosphatase complex between these two cytoskeletal components (Fig.3). SPECC1L and the MYPT1/PP1β complex likely work in concert to influence the phosphorylation status of NMII, which influences cell migration and adhesion (38, 39).

Association with adherens junctions

In epithelial cells, SPECC1L is localized to cell-cell boundaries where it likely associates with membrane-bound β-catenin and E-cadherin, both components of actin-based adherens junctions (9). Upon SPECC1L deficiency in knockdown or null cells, there is an increase in adherens junction markers β-catenin and E-cadherin, as well as abnormally elongated apico-basal electron dense regions in electron micrographs at the membrane (9). Wilson *et al.* (9) argued that these defects suggested stronger cell-cell adhesions between SPECC1L-deficient cells. Consistently, in some severely affected *Specc1l* mutant mouse embryos, these cell-cell adhesion defects lead to abnormal delamination (9) and clustering of migratory neural crest cells (40). Further, edema is observed rostro-caudally in all *Specc1l* null mutant embryos around midgestation, either as midline subepidermal blebs on the cranium or from the head all along the spine (10). Subepidermal blebs and edema are found in mutants in

the FREM/FRAS and PDGFRA-PI3K pathways (24, 41–44), which play important roles in cell-cell and cell-matrix adhesion.

Hall *et al.* observed abnormal oral adhesions between the palatal shelves and the tongue, also at midgestation (10). Pathological oral adhesions were first described in mouse mutants of *Irf6* hypomorphic alleles or of other genes in the IRF6 network (e.g., *Arhgap29, Jagged2, Grhl3*), which resulted in palate elevation delay or cleft palate (18, 45–50). These ectopic oral adhesions occurred either due to a loss or abnormality of the transient embryonic superior-most epithelial layer, called the periderm (51). The periderm is specialized such that adhesion molecules are not expressed at its apical surface, thus preventing abnormal adhesions between embryonic epithelial surfaces (45, 51). Later in gestation, the periderm is replaced by the keratinized epithelium. Hall *et al.* (10) showed that SPECC1L-deficient periderm frequently displayed adhesion molecules, including again β-catenin and E-cadherin, at its apical surface (10). However, these periderm adhesion defects in *Specc1l* null mutants appear to be transient or weak, such that they only delayed palate closure. Further, consistent with previous findings involving ectopic oral adhesions, Hall *et al.* (10) showed that SPECC1L expression was lost in *Irf6* null palatal shelf epithelium and mesenchyme. Taken together, these data suggest that SPECC1L functions downstream of IRF6 to sequester adhesion molecules away from the apical surface in periderm cells.

Interestingly, neither edema nor ectopic oral adhesions were observed in *Specc1l-ΔCCD2* mutants (6), suggesting that CCD2 interaction with microtubules may be less important for SPECC1L association with adherens junctions. However, it is important to note that while *Specc1l* null mutants do not show tissue movement and fusion defects, they are developmentally more severely affected and are embryonic lethal. It is likely that these epithelial adherens junction defects underlie the phenotypic severity and lethality in the *Specc1l*-null mutants.

Modulation of collective cell migration of attributes

Saadi *et al.* (8) initially showed that SPECC1L-deficient U2OS osteosarcoma cells performed poorly in wound-closure assay. Wilson *et al.* (9) later showed that these *SPECC1L*-kd U2OS cells, when plated at high density, formed abnormally increased cell-cell adhesions. Goering *et al.* (6) then showed that both *SPECC1L*-kd U2OS cells and SPECC1L-deficient primary MEPM cells formed narrower streams under regular culture conditions but at high density. Further, in wound-repair assays, these SPECC1L-deficient cells showed poor directionality of movement. Both cell stream formation and cell directionality are attributes of collective cell movement. Interestingly, these defects in cellular attributes could be largely rescued with the administration of PI3K-AKT pathway activators (see below).

Other potential cellular roles

An early finding of Wilson *et al.* (9) was that of downregulation of PI3K-AKT pathway upon SPECC1L deficiency in cells. The reduction in PI3K-AKT signaling was also consistent with increased E-cadherin levels and increased adherens junction markers. This relationship between PI3K-AKT pathway and adherens junctions has been well-

characterized in cancer cells, where increased PI3K-AKT signaling is associated with reduced E-cadherin and adherens junctions leading to increased metastatic features (52, 53). Consistent with a functional role of reduced PI3K-AKT signaling in SPECC1L deficiency, treatment with PI3K-AKT activators rescued several cell adhesion and migration defects of *Specc1l kd* and mutant MEPM cells (9, 10). Interestingly, while activated phospho-S473-AKT was reduced in SPECC1L-kd cells, this reduction was commensurate to pan-AKT reduction (9). In *Specc1l* mouse mutant tissue, AKT2 was more reduced than AKT1 or AKT3 (10). Regardless, reduction in pan-AKT (or AKT2) levels, rather than phospho-AKT, is rare and likely regulated by AKT stability (9). AKT activation requires localization to the membrane. Prevention of this step leads to instability and degradation of AKT (54, 55). Whether SPECC1L plays a direct role in maintaining AKT stability or whether reduced AKT stability is secondary to the membrane defects upon *Specc1l* deficiency remains to be determined.

Confocal imaging of SPECC1L cellular expression indicates that SPECC1L is also present in the nucleus. Recent domain annotations using the Eukaryotic Linear Motif (ELM) resource (56) suggested that SPECC1L may indeed possess both a nuclear localization signal (NLS) and a nuclear export signal (NES) (Fig.1). The veracity of these domains and their function remains to be determined. However, as mentioned previously, SPECC1L does associate with the mitotic spindle microtubules (8) and SPECC1L deficiency leads to abnormal cell division (7, 57). Whether the nuclear expression of SPECC1L is related to its role in cell division or is an entirely independent function is unclear. A future experiment may be to observe SPECC1L dynamics in live cells to parse out the relationships of its diverse roles during different cellular processes.

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Perspective

- Structural birth defects are observed in approximately 3% of all babies born. Regulation of cytoskeletal actin and NMII organization, and thus actomyosin forces, in embryonic development is critical in understanding the etiology of many structural birth defects.
- SPECC1L is a novel cytoskeletal scaffolding protein that appears to modulate propagation of actomyosin forces. SPECC1L associates with actin, NMII, myosin phosphatase, microtubules and adherens junction components.
- Identification and characterization of SPECC1L intracellular dynamics, protein interactors and mutant mouse alleles will help elucidate the regulation of embryonic tissue movement, adhesion and fusion processes.

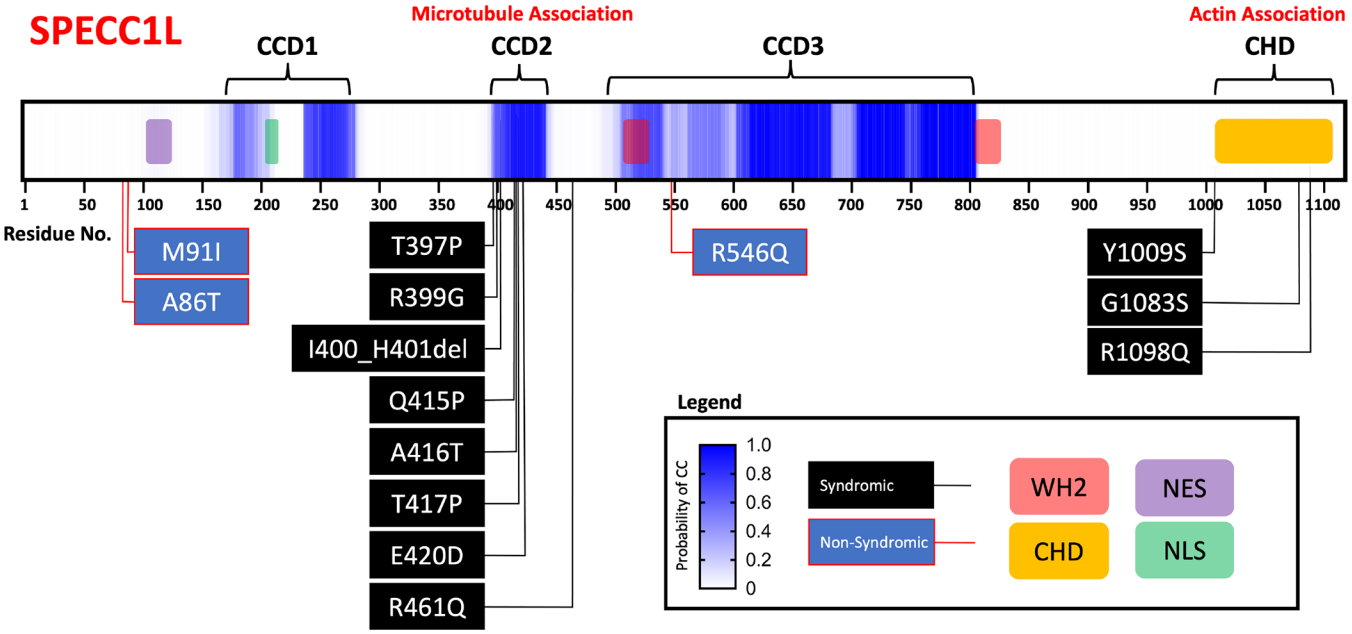

Figure 1: Schematic of SPECC1L protein with known domains, motifs, and human mutations.

Schematic shows SPECC1L protein with human mutations identified in patients with *SPECC1L-related syndrome* (black) or with non-syndromic cleft lip and/or palate (blue) (8, 10–14, 58). The probability that each amino acid is part of a coiled-coil (CC), as predicted by MultiCoil (59), is shown as a color gradient (white = 0% CC, blue = 100% CC). Three broad CC Domains (CCDs) can be predicted based on the MultiCoil probabilities. Additional selected motifs using Eukaryotic Linear Motif (ELM) resource (56) shown, include Nuclear Export Signal (NES, purple), Nuclear Localization Signal (NLS, green), WASP-Homology 2 (WH2) domains (pink), and Calponin Homology Domain (CHD, yellow).

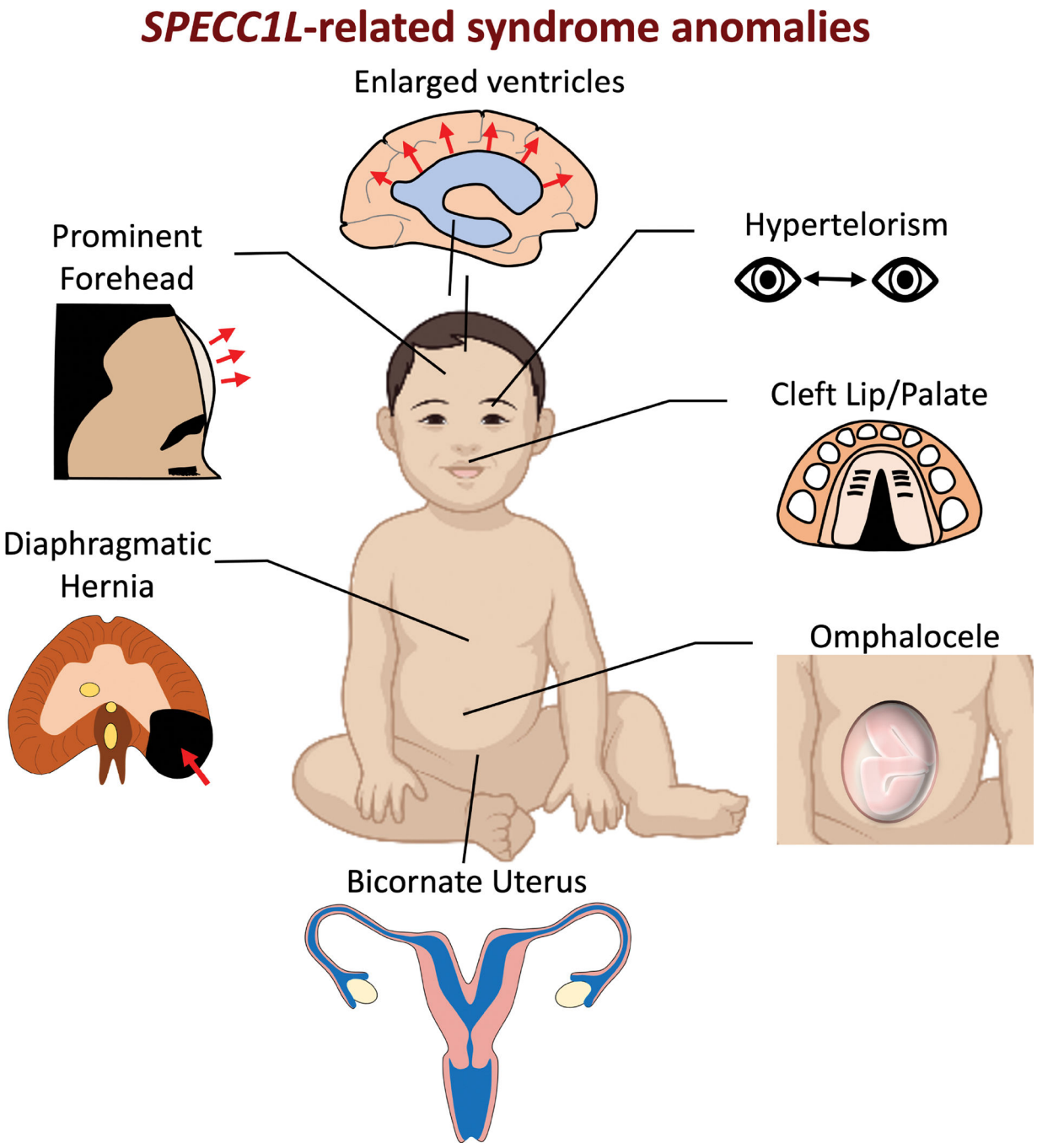

Figure 2: Developmental defects associated with *SPECC1L-related syndrome*.
Some of the phenotypes commonly associated with *SPECC1L-related syndrome* are depicted. Clockwise from right: orbital hypertelorism (~97%), cleft palate (~23%), omphalocele (~48%), bicornate uterus (100% among females), diaphragmatic hernia (~23%), prominent forehead (~86%), and enlarged ventricles (~67%). Phenotypes depicted here are not exhaustive. For additional information, please review Bhoj *et al.* (2019) (12). Schematic partially created with BioRender.com.

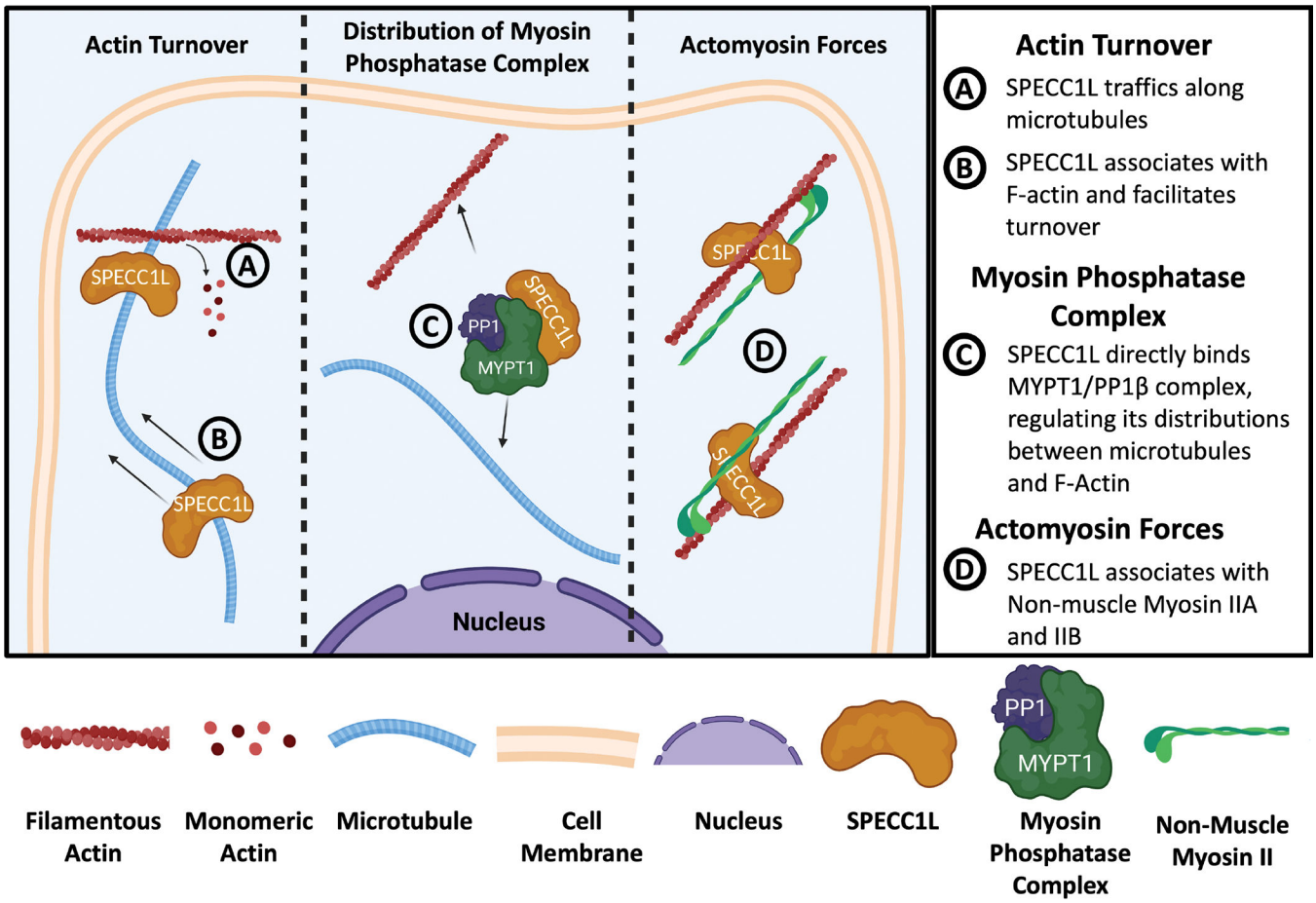

Figure 3: Cellular Functions of SPECC1L.

Schematic representation of known and proposed SPECC1L roles in the cell. SPECC1L traffics in the cell via its association with microtubules (A), and likely facilitates filamentous actin (F-actin) turnover (B). SPECC1L physically interacts with non-muscle Myosin II (NMII) phosphatase complex (MYPT1), and likely regulates distribution of the NMII phosphatase complex between F-actin and microtubules (C). SPECC1L also physically interacts with NMIIA (MYH9) and NMIIB (MYH10), directly influencing actomyosin organization within the cell (D). Schematic partially created with BioRender.com.