**A combined in silico and in vivo approach to the structure-function annotation of SPD-2 provides mechanistic insight into its functional diversity**

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**ABSTRACT**

Centrosomes are organelles that function as hubs of microtubule nucleation and organization, with key roles in organelle positioning, asymmetric cell division, ciliogenesis, and signaling. Aberrant centrosome number, structure or function is linked to neurodegenerative diseases, developmental abnormalities, ciliopathies, and tumor development. A major regulator of centrosome biogenesis and function in *C. elegans* is the conserved Spindle-defective protein 2 (SPD-2), a homolog of the human CEP-192 protein. CeSPD-2 is required for centrosome maturation, centriole duplication, spindle assembly and possibly cell polarity establishment. Despite its importance, the specific molecular mechanism of CeSPD-2 regulation and function is poorly understood. Here, we combined computational analysis with cell biology approaches to uncover possible structure-function relationships of CeSPD-2 that may shed mechanistic light on its function. Domain prediction analysis corroborated and refined previously identified coiled-coils and ASH (Aspm-SPD-2 Hydin) domains and identified new domains: a GEF domain, an Ig-like domain, and a PDZ-like domain. In addition to these predicted structural features, CeSPD-2 is also predicted to be intrinsically disordered. Surface electrostatic maps identified a large basic region unique to the ASH domain of CeSPD-2. This basic region overlaps with most of the residues predicted to be involved in protein–protein interactions. In vivo, ASH::GFP localized to centrosomes and centrosome-associated microtubules. Our analysis groups ASH domains, PapD, Usher chaperone domains, and Major Sperm Protein (MSP) domains into a single superfold within the larger Immunoglobulin superfamily. This study lays the groundwork for designing rational hypothesis-based experiments to uncover the mechanisms of CeSPD-2 function in vivo.

**Abbreviations:** AIR, Aurora kinase; ASH, Aspm-SPD-2 Hydin; ASP, Abnormal Spindle Protein; ASPM, Abnormal Spindle-like Microcephaly-associated Protein; CC, coiled-coil; CDK, Cyclin-dependent Kinase; Ce, Caenorhabditis elegans; CEP, Centrosomal Protein; CPAP, centrosomal P4.1-associated protein; D, Drosophila; GAP, GTPase activating protein; GEF, GTPase guanine nucleotide exchange factor; Hs, Homo sapiens/Human; Ig, Immunoglobulin; MAP, Microtubule associated Protein; MSP, Major Sperm Protein; MDP, Major Sperm Domain-Containing Protein; OCR-1, Golgi endocytic trafficking protein Inositol polyphosphate 5-phosphatase; PAR, abnormal embryonic PArTitioning of the cytosol; PCM, Pericentriolar material; PCMD, pericentriolar matrix deficient; PDZ, PSD95/Dlg-1/zo-1; PLK, Polo like kinase; RMSD, Root Mean Square Deviation; SAS, Spindle assembly abnormal proteins; SPD, Spindle-defective protein; TRAPP, TRAnsport Protein Particle; Xe, Xenopus; ZYG, zygote defective protein.

**Introduction**

Centrosomes are organelles with key roles in the positioning of other organelles in the cell and function as hubs for microtubule nucleation and organization. They are composed of a pair of microtubular centrioles immersed in a protein matrix known as the pericentriolar material (PCM). The centrioles and the PCM are codependent for many of the centrosome functions but have distinct roles and largely associated with different sets of proteins [1,2]. An exception is the *C. elegans* spindle-defective protein 2 (CeSPD-2) protein, a homolog of the human CEP-192 protein. CeSPD-2 localization to the centrosome or
exclusively to the centrioles is likely relevant to its dual role on centriole duplication and centrosome maturation [3,4].

The first mitosis of the C. elegans embryo has been a central model for understanding centrosome bio-
genesis, function, and structure [5]. At fertilization, the sperm provides a pair of engaged centrioles that separate, duplicate, and recruit PCM proteins present in the oocyte’s cytosol. SPD-2 is required throughout the assembly of the maternally contributed PCM [1]. In the zygote, SPD-2 localizes to the sperm centriole pair and around the sperm DNA [6]. The earliest detectable PCM proteins around the sperm-provided centrioles are the coiled-coil proteins PCM deficient-1 (PCMD-1), SPD-5, SPD-2, and the microtubule anchoring protein γ-tubulin. SPD-2 and SPD-5 are mutually dependent for their localization to the PCM, and PCMD-1 is required for the recruitment of SPD-5 [3,7]. SPD-2 and γ-tubulin accumulation on the centrosome is partially dependent on the Aurora A Kinase (AIR-1 in C. elegans) and cytoplasmic dynein dhc-1 [3]. As the embryo enters mitosis SPD-2 and SPD-5 promote PCM assembly and centrosome maturation [3]. SPD-2 recruits polo-like kinase-1 (PLK-1) and Aurora kinase A (AIR-1), which are required for maturation [8,9]. At metaphase PCM components are restructured. PLK-1 phosphorylates SPD-5 [10] and this initiates polymerization of PCM and the formation of a γ-tubulin ring surrounded by a microtubule ring [11,12].

As the PCM volume and γ-tubulin increase [13,14], the sperm-provided centrioles separate and start to assemble daughter centrioles by sequentially recruiting maternally provided structural and regulatory centriolar components [15–17]. This process is dependent on PCM proteins [2]. Six proteins are required for centriole assembly; the likely ortholog of Polo like kinase 4 (PLK4) in mammals Zygote defective 1 protein (ZYG-1) [18], and coiled-coil proteins: SPD-2 [3,4,15] and the other Spindle assembly abnormal proteins SAS-4 [19], SAS-5 [20], SAS-6 and SAS-7 [21]. SAS-7 directly binds to and recruits SPD-2 to the emerging daughter centrioles [21]. Although CeSPD-2 and its human ortholog CEP192 are essential for centriole duplication [4,22,23] it is not clear whether this is true for the Drosophila orthologue, DsSPD-2 [24,25].

As the sperm provided centrioles duplicates, the anteroposterior polarity of the zygote is established. This asymmetry breaking depends on the male pronucleus associated centrosome and possibly the centrosome associated microtubules [26–30]. Therefore, polarity in the C. elegans embryo indirectly depends on the centrosome maturation roles of CeSPD-2 and SPD-5. SPD-2 and SPD-5 have also been proposed to have direct roles in breaking symmetry in the embryo [31–33], but this possibility remains contentious.

CeSPD-2 is an excellent entry point for uncovering mechanisms underlying centrosome function and inheritance, because of its multiple roles and intermolecular interactions, and because its poorly understood regulation and mechanisms of function. We took a mostly in silico approach to predict structural features and domains in CeSPD-2 to base future inquiries about its regulation and function. We report that despite being predicted to be a largely intrinsically disordered protein, CeSPD-2 has several functional domains including previously unreported GEF, Ig-like, and PDZ-like domains, as well as a fourth coiled coil. In addition, this thorough analysis provided a refined prediction of previously reported coiled coils and the Aspm-SPD2-Hydin (ASH) domain of CeSPD-2. The modeled three-dimensional structure of the ASH domain reveals that it is a member of a structural superfold that includes Major Sperm Protein (MSP), PapD, and usher-chaperone domains. The CeSPD-2 ASH domain localizes to the centrosome in-vivo as previously shown in vitro for other ASH/PapD domain containing proteins [34–37]. Based on previous reports and our findings, we speculate on how the domains and structural features of CeSPD-2 agree with its roles, and hint at possible mechanisms of function and its regulation.

**Results**

**CeSPD-2 is a multidomain protein with an intrinsically disordered N-terminus**

The predicted secondary structure of C. elegans SPD-2 protein sequence (NP_492414.1) (Figure S1) suggests that it is 50% unstructured. The N-terminal half of CeSPD-2 including the first
460 residues contains only five to seven \( \alpha \)-helices (Figure 1 and S1) and is predicted to be intrinsically disordered (72\%) (Figure 1). CeSPD-2 was previously reported to have three coiled-coils (CC), similarly located to our prediction of CC1 (65–82), CC2 (111–138), and CC3 (291–322) (Figure 2 and S1) [3]. Our analysis also identified an additional fourth coiled-coil region, CC4 (368–400). CC3 and CC4 lie within a previously unpredicted RhoGEF-like domain motif (258–467) (Figure 1). This domain is most similar to the RhoGEF domain in the hypothetical Rom1 protein from \textit{Encephalitozoon cuniculi} (14–218) that has sequence similarity to the DH domains of RhoGEFs Rom1 and Rgf1 in \textit{S. cerevisiae} and \textit{S. pombe}, respectively. In contrast to the largely unstructured N-terminus, the C-terminal half of CeSPD-2 is predicted to contain several secondary structure signatures indicating hallmarks of protein domains. It is predicted to contain a previously reported [38] ASH domain (475–564), followed by previously unreported Immunoglobulin (Ig)-like (593–660) and PDZ-like (695–820) domains (Figure 1). ASH domains are remote homologs of the immunoglobulin (Ig)-like seven-stranded beta sandwich fold superfamily [38]. The Ig-like domain is made up of six anti-parallel \( \beta \)-sheets in a sandwich conformation reminiscent of an immunoglobulin fold (Figures 1) [39]. The PDZ-like domain contains the carboxylate-binding loop that includes the highly conserved R/K-X-X-X\( -\)G\(-\)\( \Phi \) motif (R743-F749) [40,41].

**ASH domains show low primary sequence conservation but have highly conserved structure**

A PSI BLAST search (E-value threshold = 0.005) using the CeSPD-2 ASH domain identified only three significant sequence matches: the predicted \textit{Caenorhabditis} species homologs of SPD-2 from \textit{C. briggsae}, \textit{C. remanei}, and a hypothetical protein from the hookworm parasite \textit{Necator americanus} (Table S1). The third iteration identified seven additional distantly related protein sequences at or above 0.05 E-value threshold. These include \textit{Lingula anatine} orthologs of SPD-2, a cilia and flagella-associated protein 74 from \textit{Spartus aurata}, and 5 other predicted proteins of unknown function or proteins with only preliminary sequences from the first round of shotgun sequencing (Table S1).
Figure 2. Comparison of sequences of ASH domains from centrosomal proteins.

(a) Multiple sequence alignment of the ASH domains for centrosomal proteins from *C. elegans*, *Xenopus*, *Drosophila*, *Mus musculus* and *Homo sapiens*. The sequences shown include *Caenorhabditis elegans* [Ce] spindle defective protein-2/SPD-2; Human [Hs] hydrocephalus-inducing protein/HYDIN isoforms a/b and c/d, abnormal spindle-like microcephaly-associated protein/ASPM isoform 1/2, inositol polyphosphate 5-phosphatase/OCLR-1 isoform and centrosomal protein 192kDa/CEP192; *Xenopus laevis* [Xe] centrosomal protein/CEP192; *Mus musculus* [Mm] hydrocephalus inducing protein/HYDIN; *Drosophila melanogaster* [Dm] abnormal spindle protein/CEP192. The sequences shown include *Caenorhabditis elegans* [Ce] spindle defective protein-2/SPD-2; Human [Hs] hydrocephalus-inducing protein/HYDIN isoforms a/b and c/d, abnormal spindle-like microcephaly-associated protein/ASPM isoform 1/2, inositol polyphosphate 5-phosphatase/OCLR-1 isoform and centrosomal protein 192kDa/CEP192; *Xenopus laevis* [Xe] centrosomal protein/CEP192; *Mus musculus* [Mm] hydrocephalus inducing protein/HYDIN; *Drosophila melanogaster* [Dm] abnormal spindle protein/CEP192. The sequences shown include *Caenorhabditis elegans* [Ce] spindle defective protein-2/SPD-2; Human [Hs] hydrocephalus-inducing protein/HYDIN isoforms a/b and c/d, abnormal spindle-like microcephaly-associated protein/ASPM isoform 1/2, inositol polyphosphate 5-phosphatase/OCLR-1 isoform and centrosomal protein 192kDa/CEP192; *Xenopus laevis* [Xe] centrosomal protein/CEP192; *Mus musculus* [Mm] hydrocephalus inducing protein/HYDIN; *Drosophila melanogaster* [Dm] abnormal spindle protein/CEP192.
We wanted to know how conserved the secondary and tertiary structure of ASH domains was, despite their very low primary sequence similarity. To find this, we utilized proteins with known structure and function associated with the centrosome, the cilia, and/or the flagellum, because the PSI-BLAST search yielded only putative or uncharacterized SPD-2 orthologs, predicted proteins, or proteins that are uncharacterized and/or have unknown function and structure. Specifically, we compared the CeSPD-2 ASH domain with its orthologs Centrosomal Protein 192 (Cep192) from *Xenopus laevis* [42] and *Homo sapiens* [43,44], the *Drosophila melanogaster* Abnormal Spindle Protein (ASP) [45], human Abnormal Spindle-like Microcephaly-associated Protein (ASPM) [38,46], the ciliary protein Hydin (*Homo sapiens* and *Mus musculus*) [47–49], and the *Homo sapiens* Golgi endocytic trafficking protein Inositol polyphosphate 5-phosphatase (OCRL-1) [50–52]. Sequence alignment of these ASH domains shows that, as expected, they share little sequence similarity (Figure 2A). An asparagine (N503 in SPD-2) that had been previously defined as a signature feature of ASH domains, is the most highly conserved residue (Figure 2A) [38]. Other highly conserved residues include an N-terminal phenylalanine (F486 in CeSPD-2 but absent in XeCEP192), and a proline (P534 in SPD-2, absent in ASP and HsCEP192). In pairwise comparison, CeSPD-2 ASH has higher sequence similarity (33.33%) to the ASH domain of the non-orthologous ASPM protein from humans (Figure 2B) than to its orthologs, CEP192 from *Xenopus* (32.39%) and humans (26.88%). Interestingly, the *Xenopus* CEP192 ASH domain shares more similarity with the CeSPD-2 ASH domain than it does with the human CEP192 ASH domain (30.98%). Despite having little primary sequence similarity, the secondary structure of these centrosome proteins ASH domains appear to be well conserved. For instance, the 7 β-strands predicted in the CeSPD-2 ASH domain are located at similar positions to β-strands previously determined by X-ray diffraction in the ASH domain from OCRL-1 (3QIS[52]) (Figure 2A). To assess conservation of the tertiary structure of the centrosome protein’s ASH domain, we first generated a 3D model for the ASH domain in CeSPD-2, ASPM, and ASP (Figures 3 and 4A). The top ranked 3D model for the CeSPD-2 ASH domain was produced by LOMETS [53] using the C-terminal PapD-like domain of human Hydin as the template (Figure 3A). Although the human Hydin ASH/PapD domains and the CeSPD-2 ASH domain share only about 20% sequence similarity (Figure 2B), these superimposed ASH and Pap-D domains show overall high structural similarity (RMSD = 1.125 Å) (Figure 3A). Further support for the modeled 3D structure is that the most conserved residues (F5, N22, and P53) are in similar location and orientation in the CeSPD-2 and OCRL-1 ASH domains (Figure 3B). Although the topology of Hydin ASH/Pap-D and CeSPD-2 ASH β-sheets differ, both domains form an antiparallel β-sandwich conformation with a predicted classic type parallel β-bulge [54] similarly located in CeSPD-2 ASH and in Hydin PapD-like domain (Figure 3C). The β-bulge in the modeled CeSPD-2 ASH domain forms between residues A4, F5 on β-sheet 1 and W91 on β-sheet 7 (Figure 3C). β-bulges often form between a residue in a β-sheets and two residues from an antiparallel β-sheet, and slightly increases the usual right-handed strand twist of the β-sheets. β-bulges are thought to affect the orientation of a binding site, help with dimerization, and help with accommodating single residue mutations [54,55].

We also generated 3D model predictions for the ASH domains of the centrosome/cilia/flagella proteins ASPM and ASP from the previous alignment in Figure 2A (Figure 4A). The top template selected for the ASP ASH domain 3D model was the OCRL-1 ASH domain in complex with Rab8 (3QBT [52]). Despite showing high structural similarity (RMSD = 2.285 Å), OCRL-1 and ASP ASH domains shared only 20.40% sequence

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protein. Amino acids with 70% equivalency are labeled in red and boxed in blue. Highly conserved residues are highlighted in red. The CeSPD-2 and the OCRL-1 ASH domains secondary structures are diagrammed in the context of their primary sequence, with β-sheets shown as green arrows and the α-helices shown as black corkscrews. (b) Sequence Similarity of ASH domains is depicted in a similarity matrix comparing the nine observed ASH domains.
Figure 3. Comparison between the three-dimensional model of the SPD-2 ASH domain and the known structure of ASH domains of HYDIN or OCRL-1.
similarity (Figure 2B). Interestingly, the C-terminal domain of a PapD-like domain from a protein of unknown function in *Porphyromonas gingivalis* (2QSV [56]) was selected as the best template for the ASH domain of ASPM (RMSD = 3.0 Å) (Figure 4A).

**ASH domains conserved Immunoglobulin-like structural fold overlaps with that of homologous superfamilies of PapD, MSP and Chaperone Domains**

Because the ASH domains from proteins with centrosome/cilia/flagella function or localization show low sequence conservation but higher secondary structure conservation, we performed a DALI structure-based search with the CeSPD-2 ASH domain 3D predicted structure. This search identified 10 domains of known structure analogous to CeSPD-2 ASH. These include at least three PapD domains, three MSP domains, and two bacterial pilus chaperone-usher proteins (Figure 4B and Table S1). The C-terminal PapD-like domain of HsHYDIN protein (2E6J [47]; Z-score: 15.4) and an ASH domain of HsCEP192 protein (6FVI [43]; Z-score: 11.1), were the two closest neighbors for the CeSPD-2 ASH domain (Figure 4B and Table S1). This is consistent with the HsHYDIN PapD domain being the chosen template for the top ranked SPD-2 ASH 3D model (Figure 4A). The domains identified based on structure-similarity show little to no conservation of primary sequence between ASH domains but highly conserved secondary structure alignment (Figure 4B), as observed in the sequence alignment of ASH domains from centrosome/cilia/flagella associated proteins (Figure 2A). Superimposition of the modeled CeSPD-2 ASH domain and the 10 closest structural domain neighbors, shown in Movie S1, provides a clear visual confirmation of how closely related the structure of these domains is. All 10 3D structures superimposed with low RMSD (≤2.1 Å) indicating a common structural fold (Figure 4B). Together, the DALI search for structurally similar domains to the CeSPD-2 ASH 3D model and the choice of PapD domains as templates for 3D top models for ASH domains are evidence that ASH, PapD, MSP, and chaperone-usher domains belong to the same Superfold.

**CeSPD-2 ASH has a unique biochemical character that may have functional significance**

To identify possible functional regions within the ASH superfold, we generated surface electrostatic potential maps for the predicted ASH domains of the centrosomal proteins CeSPD-2, ASP, and ASPM, and the domains of known structure that served as their 3D prediction structural templates HYDIN, OCRL-1, and *Porphyromonas gingivalis* W8354 protein (Figure 4A). The electrostatic profile of CeSPD-2 ASH shows a large basic groove that is formed by residues K1-C7 and Y71-N93 (Figure 4A). A much smaller basic groove is found at a similar location in the Hydin PapD-like domain (2E6J), and this groove is significantly reduced or absent in ASP, OCRL-1, ASPM, and 2QSV (Figure 4A).

To ask whether the basic groove might be involved in functional interactions, we considered whether the residues predicted to form protein–protein or protein–nucleic acids in the ASH domain overlap with the basic residues (Figure 5A–C). We found that half of the residues in the basic groove (15/30) are predicted to be involved in protein–protein interactions (Figure 5A and 5B), and under a third of the

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(a) Superposition of the modeled CeSPD-2 ASH domain with the human Hydin C-terminal PapD-like domain template (PDB ID: 2E6J [47]) and sequence alignment based on the superposition. Residues with white letters that are highlighted in red are identical in both domains. Red letters in unfilled blue boxes correspond to similar residues. (b) Overlays of SPD-2 ASH (orange) with the unbound structure of the ASH domain from OCRL-1 (3QIS [52]) with highly conserved residues highlighted and labeled. Images were produced with Pymol [117]. (c) The three-dimensional ribbon diagram of the C-terminal Hydin PapD domain and the predicted CeSPD-2 ASH domains are shown next to their topology diagrams. The β-bulges in SPD-2 and Hydin are magnified, and participating residues are highlighted in purple. The arrows in the topology diagram represent β-sheets and their orientation. Cylinders represent α-helices, and the yellow boxes denote the N- and C-terminus of the domains. Residue numbers are included at the N- and C-terminus of each secondary structure.
Figure 4. Electrostatic and structural similarities between the ASH domains from SPD-2 and other ASH domain containing proteins.
basic residues (7/30) are predicted protein–RNA interaction sites (Figure 5A–C). There are 19 overlapping residues within the ASH domain that are predicted to bind both protein and RNA, these include the 7 residues predicted to bind RNA located in the basic groove. Comparison of contact site predictions for several ASH containing proteins associated with centrosome/cilia/flagella function or localization and CeSPD-2 ASH (Figure S2) show no overlap between the predicted sites from ASH in SPD-2 and the ASH domains from either Homo sapiens or Xenopus CEP192 (SPD-2 homolog) nor HYDIN (top 3D template for SPD-2). Therefore, neither the electrostatic potential maps nor the contact site predictions define a general ASH domain-specific functional region.

The DALI search for folds analogous to the SPD-2 ASH domain identified bacteria pil common chaperones and chaperone usher proteins (Table S1). These chaperones have two Ig-like or PapD domains oriented to one another in a functional structure that resembles a boomerang shape[39]. Because several of the previously identified proteins have two or more Ig-like and/or ASH/PapD domains (e.g. HYDIN and CEP192), and CeSPD-2 has an Ig-like domain adjacent to the ASH domain, we wondered whether they also may form a boomerang structure analogous to the common and usher chaperones in bacteria. The 3D model of the CeSPD-2 ASH and Ig-like domains together also forms a boomerang-shape structure very similar to the bacterial chaperones identified in the DALI search (Figure 5D) [56]. Interestingly, the boomerang shape brings together several basic regions within the CeSPD-2 primary sequence. The basic region on the surface of the ASH domain extends into the cleft between the two domains, to the outward facing surface of the first β-sheet of the Ig-like domain (S2-K32), into the cavity between the first β-sheet and the second β-sheet (K57-V63), and ends at the short α-helix (M37-Q39) (Figure 5D).

The CeSPD-2 ASH domain localizes to centrosomes

ASH domains have been proposed to have microtubule binding function [38] and have been also shown to localize to centrosomes and cilia basal bodies in vitro [34,37,38,57]. To test whether the ASH domain of CeSPD-2 localizes to the centrosome, we expressed the ASH domain of CeSPD-2 as a fusion protein with GFP (ASH::GFP) in vivo (Figure 6). Failure to generate a strain expressing GFP from a MosSCI insertion of an ASH::GFP construct driven by a germline promoter (Personal communication T. Mikeladze-Dvali) suggested that expression of the ASH::GFP protein might be toxic. We therefore expressed the ASH::GFP construct in a few cells of the animal and transiently upon induction. Strains expressing an extrachromosomal array of ASH::GFP driven by a germline-specific promoter yielded GFP expressing strains (see materials and methods) [58,59]. Further, suggesting that expression of ASH::GFP might be toxic was the very low germline transmission (≤10%) of the construct in the two strains obtained. In these strains, GFP was detected on the centrosomes and centrosome-associated microtubules in cells of embryos undergoing mitosis (Figure 6G–I). We also generated strains carrying

(a) The modeled structure of the SPD-2 ASH domain, the Human Hydin PapD-like domain (PDB ID: 2E6J [47,121]), the Human OCR1-1 ASH domain (PDB ID: 3QBT_B [51]), the Drosohila ASP ASH domain, and Human ASPM ASH domain are shown next to their surface electrostatics profiles mapped on to the models. The basic residues in CeSPD-2 and Hydin are highlighted in blue on the ribbon model. Structures for ASP and ASPM were modeled using OCR1-1 ASH when bound to Rab8A (3QBT_B [51]) and a protein of unknown function from P. gingivalis W83 (2QSV [121]) as templates, respectively. Three additional views of the electrostatic potential rotated clockwise by 90°, 180° and 270° are shown. Electrostatic images produced with APBS-PDB2PQR server and PyMOL [117] representing basicity in blue and acidity in red (+5 to −5 kT/e). (b) Structure-based sequence alignment of structurally similar domains to CeSPD-2 identified using DALI [108]. Primary sequence and secondary structure of the domains identified as having similar tertiary structure to CeSPD-2 ASH are shown in an alignment. Residues in the primary sequence in blue are the most conserved, residues in red show the lowest conservation and residues in green are those with intermediate conservation. Secondary structure is annotated with β-sheets as blue letter Es and the loops between them in green letter Ls. Dash line arrows also show the alignment of β-sheets between the identified sequences structurally similar to CeSPD-2 for comparison with Figure 2A alignment with distant ASH sequence alignment.
Figure 5. Predicted contact sites for SPD-2 ASH domain.

(a) Surface electrostatic profile of SPD-2 ASH with the corresponding residues that form the basic groove labeled in blue on the sequence below. (b) Predicted structure-based protein–protein interaction sites are highlighted in orange on both the backbone of the modeled CeSPD-2 ASH domain and its sequence. (c) Predicted RNA binding sites on the SPD-2 ASH domain are highlighted in purple in both the backbone structure and sequence. (d) Model and electrostatic profile of the tandem ASH and Ig-like domains. The three-dimensional ribbon structure and predicted electrostatic profile of the SPD-2 ASH (gold) and Ig-like (blue) domain models assembled into a single unit. Blue represents basicity and red represents acidity (+5 to –5 kT/e).
Figure 6. Overexpressing the SPD-2 ASH domain in C. elegans embryos.

(a-e) Images generated from partial z-stack projections of embryos expressing the translational fusion of the ASH domain and GFP under the control of an inducible heat-shock promoter. ASH::GFP is expressed from an extrachromosomal array that is inherited by a proportion of the embryos. Arrowheads mark ASH::GFP foci or aggregates. Scale bar is 15 μm. (a-c) Fluorescent (a), Differential Interference Contrast (DIC) (b) and merged (c) images of two embryos submitted to the same heat shock protocol. The embryo on the right expresses GFP whereas the embryo on the left does not express ASH::GFP, likely because it did not inherit the extrachromosomal array including the ASH::GFP construct. (d-f) A pair of embryos expressing ASH::GFP imaged by DIC (d) and fluorescence microscopy (e) merged fluorescence and DIC images. (f) Digital magnification of a region of embryo in E. (g-i) Images of embryos expressing MCherry::H2B (red fluorescent Histone marker) and ASH::GFP driven by the germine promoter Pie-1. ASH::GFP localizes broadly to the centrosome, spindle microtubules (g), and centrosome associated microtubule asters (h). Scale bar is 10 μm. Arrows mark centrosomes. (i) Digital magnification of a region of the embryo in H. Scale bar is 5 μm.

A heat-shock inducible ASH::GFP transgene (see materials and methods) [58,59]. Brief heat-shock treatment induced expression of GFP that was detected as a single focus adjacent to the interphase nuclei (Figure 6A-C). Longer heat-shock treatments resulted in cytosolic GFP aggregates of variable size (Figure 6D-E).

Discussion

CeSPD-2 is a bi-functional protein, with essential roles in centriole duplication and centrosome maturation. Although protein interactions required for each of the SPD-2 functions have been identified, the molecular mechanisms of its interactions and its regulation are largely unknown. For instance, it is unknown how specific interactions with SPD-2 that take place either exclusively at the centrioles or at the PCM are regulated.

CeSPD-2 intrinsic disorder may be important for its multifunctionality

We found that CeSPD-2 is highly disordered (50%), with most of the disordered regions being in the N-terminal half of the protein (72%) (Figure 1). Many intrinsically disordered proteins display phase transition behaviors [60]. One possibility is that CeSPD-2 might aid in the regulation of the phase-separation properties of the PCM [61]. Consistent with this is the finding in PCM reconstitution experiments that CeSPD-2 and PLK-1 have been shown to either initiate or enhance SPD-5 formation of spherical condensates.
characteristic of proteins with phase transition properties [12]. Additionally, similarly to regulators of transcription, translation, and cell cycle with disordered regions that act as hubs for different protein interactions [59,60], the highly disordered N-terminus of CeSPD-2 might provide the conformational plasticity necessary to accommodate alternative binding partners that operate in different processes [14,62–64]. For example, CDK targets often contain multiple phosphorylation sites within intrinsically disordered regions that allow for alternate phosphorylated outputs that produce different responses [65,66]. The N-terminus of CeSPD-2, expected to be intrinsically disordered, has predicted and experimentally confirmed CDK and PLK-1 phosphorylation sites (Figure 1) [4,14,67]. This region is where PLK-1 binds to promote centrosome maturation [63,68] and ZYG-1/PLK4 binds to initiate centriole duplication [23,64]. We speculate that the intrinsically disordered N-terminus might allow alternate CDK (or other kinase) phosphorylated outputs that prime CeSPD-2 for either PLK-1 and/or ZYG-1/PLK-4 binding.

The SPD-2 domain[4] ASH and Ig-like domains may be important for SPD-2 regulation.

Together, the Ig-like and ASH domains make up most of the “SPD-2 domain”, previously defined based on its shared similarity with the DsSPD-2 and HsCEP192 orthologs [4]. The DALI structure-based search with the SPD-2 ASH 3D model identified bacterial pili PapD/usher chaperones domains as closely related folds. These chaperones are two Ig-like or PapD domains oriented toward each other that form a bilobed boomerang-like structure important for protein interactions during pilus assembly [69]. We generated a 3D model of the SPD-2 ASH domain together with the Ig-like domain because of the similarity of the SPD-2 ASH domain to the PapD chaperones and because several proteins have more than one domain from the Immunoglobulin superfamily (e.g. CEP192, OCRL-1 and HYDIN). This 3D model strongly resembles the boomerang-shape of the PapD-like chaperones in bacteria (Figure 5D). The electrostatic map of the ASH and Ig-like domain 3D model predicts a large basic region that extends from the ASH domain (K1-C7; Y71-N93) to the boomerang shape cleft (S2-K32) and a large region of the exposed surface of the Ig-like domain (M37-Q39; K57-V63). This boomerang structure brings together and exposes several of the primary sequence basic regions in SPD-2 (Figure 5B). Interestingly, Shimanovskaya and colleagues (2014) [64] proposed that ZYG-1 exclusive binding to the acidic N-terminus of CeSPD-2 localized at the centrioles (and not at the cytosol or PCM) might be regulated via intramolecular electrostatic interactions with one or several basic regions within SPD-2. Based on their proposal and the reported centriole duplication phenotypes of phospho-mutations within the ASH domain in polypliod intestinal cells [67], we speculate that the boomerang structure might provide a mechanism to discriminate between cytosolic (or PCM) and centriolar CeSPD-2.

Identification of the ASH/PapD Superfold

Two main observations support our designation of the ASH/PapD superfold. First, top ranked 3D models for SPD-2’s and ASPM’s ASH domains utilized as templates PapD folds from the Hydin and W3 proteins with overall high structural similarity (RMSD < 2 Å) (Figures 3A and 4A). The SPD-2 ASH 3D model using Hydin PapD as template is also supported by the similar orientation and location of conserved amino acids in the ASH domains of CeSPD-2 and the distantly related protein OCRL1 (Figure 3B). Second, the top 10 matches in an unbiased search for folds structurally similar to CeSPD-2 ASH using DALI identified the ASH, PapD and chaperone usher bacterial pilus, and MSP domains. Despite MSP domains only sharing 11% sequence similarity with PapD/ PapD-like domains, they have been classified as members of the PapD-like superfamily [70]. Likeness to PapD chaperone domains [71] is thought to be due to the potential of MSPs to bind interfaces used for filament biogenesis and to alter the rate of filament formation [72]. Here we propose that the ASH domain, the PapD-like domain, the MSP domain, and common and usher pili chaperones domains from bacteria belong to
the same superfold within the larger Immunoglobulin superfamily. Currently, there is not sufficient data to assign the Ig-like domain in CeSPD-2 and other ASH/PapD containing proteins to its own structural family.

**ASH domains contact site predictions and localization to the centrosome**

ASH domains have been proposed to have microtubule binding function [38] and have been shown to localize to centrosomes and cilia basal bodies in vitro [34,37,38,57]. Our observations corroborate CeSPD-2 ASH localizing to the centrosome and centrosome associated microtubules in vivo. This is consistent with prior observations that mutations in ASH domains impair CeSPD-2 orthologue CEP192 localization to the centrosome and cilia [3,73], and similar observations for other ASH domain containing proteins in vitro [34,45,57,73,74]. However, further studies are needed to establish the minimum requirement for its localization pattern of the ASH domain, given that localization might be dependent on its expression level and its expression might be toxic (Figure 6). It might be useful to compare the localization of ASH on its own and together with the Ig-like domain, since other centrosome/cilia ASH proteins also have additional Ig-like, ASH, or PapD domains (e.g. HYDIN and CEP192) and the Ig-like domain and ASH in SPD-2 is predicted to form a boomerang shape analogous to the PapD and usher chaperones in bacteria pili that bind actin-like fibers.

It was slightly surprising that the predicted electrostatic surface and/or protein interacting residues of ASH domains from centrosomal, cilia, and flagella proteins did not reveal any shared properties that could be associated with their localization or microtubule binding properties. Are there shared properties that promote ASH/PapD folds to associate with filaments/microtubule containing structures? One possibility is that the identification of shared chemical properties is clouded by specific additional protein interaction binding requirements of each member of this superfold [51,75]. Alternatively, only physical structure properties shared within this superfold are required for their localization.

**Testable hypotheses to uncover molecular mechanisms of SPD-2 regulation and function**

Efforts to uncover molecular mechanisms of SPD-2 function are hindered by its dual roles in centrosome duplication and maturation, two co-dependent processes. The predicted domains and structural features identified in this study can be used to design rational hypothesis-based experiments aimed at uncovering molecular mechanisms of SPD-2 regulation and function. This analysis also hints at possible explanations for the functional diversity of SPD-2, because it predicted features often associated with proteins that are multifunctional or hubs for protein interactions.

Despite the sizable number of isolated spd-2 missense mutants, there are very few mutations affecting either centriole duplication or centrosome maturation exclusively. The structures and properties predicted here provide a logical set of residues in SPD-2 to target for mutational analysis. For instance, residues in the basic region of SPD-2 ASH predicted to be involved in protein interactions might affect the localization of SPD-2 or SPD-2 binding partners at the centrosome. The twenty-five amino acid stretch within the ASH domain predicted to bind RNA might also be a good target to test possible molecular interactions with the RNA binding protein, SZY-20, that antagonizes SPD-2 and ZYG-1 [76].

The newly identified Ig-like, GEF-like, and PDZ-like domains might shed light to these domains’ roles in SPD-2 regulation and function. Several centrosome/cilia ASH proteins have more than one Ig-like/ASH/PapD domain and at least one other of these (OCRL-1) has a GTPase regulatory domain involved in vesicle trafficking. The putative GEF domain as well as the PDZ domains on their own and together in the same protein have been associated with processes that are consistent with known and putative SPD-2 functions. These include providing functional plasticity in multifunctional proteins [41], regulating of polarized exocytosis [77,78] and mediating dynamics and reorganization of the microtubule and actin cytoskeleton [79]. These domains will help identify new protein interactions or elucidate the regulation of known interactions and might provide new insight regarding a possible direct role for SPD-2.
in anteroposterior polarization of the *C. elegans* embryo [31,33,80].

**Materials and methods**

**Secondary structure prediction and sequence analysis**

The full-length *C. elegans* SPD-2 protein sequence was retrieved from the NCBI Reference Sequence (RefSeq) Protein sequence database (NP_492414.1; The *C. elegans* Sequencing Consortium, 1998) [81]. The domain architecture for SPD-2 was analyzed using SMART (Simple Modular Architecture Research Tool) [82], HHpred (v.2016) [83], Pfam [84], Prosite [85], Interpro [86], and the NCBI Conserved Domain (CD) database [87], and a final consensus domain architecture cartoon was drawn to scale using Adobe Illustrator (Adobe Inc., 2019). Predictive secondary structure analysis of the full-length protein and ASH domain-specific sequence was used to confirm domain boundaries and assess the underlying secondary structure using PSIPRED [88], PSSpred [89], SPIDER2 [90], Jpred4 [91], and SABLE [92]. Based on the domain architecture and secondary structure prediction results, the following sequence analysis was also performed for a more detailed sequence characterization: (a) Sequence disorder was predicted using Genesilico MetaDisorder [93] and PrDOS [94], and (b) Coiled-coils were predicted using COILS [95], DeepCoil [96], Paircoil2 [97], and Multicoil [98]. Potential sequence-based intermolecular interaction sites were assessed with ANCHOR [99], DisoRDPbind [100].

The ASH domain protein sequences of additional proteins were retrieved from NCBI: Human [Hs] hydrocephalus-inducing protein/HYDIN isoforms a/b and c/d (NP_001257903.1:513–613, NP_001185471.1:541–640), abnormal spindle-like microcephaly-associated protein/ASPM isoform 1/2 (NP_060606.3:37–134), inositol polyphosphate 5-phosphatase/OCRL-1 isoform a (NP_000267.2:564–678) and centrosomal protein 192kDa/CEP192 (NP_115518.3:2256–2402); *Xenopus laevis* [Xe] centrosomal protein/CEP192 (AP-36856.1:1776–1846); Mus musculus [Mm] hydrocephalus inducing protein/HYDIN (NP_766504.3:559–659); *Drosophila melanogaster* [Dm] abnormal spindle protein (NP_524488.3:25–122). Domain sequence alignments were done using the MUSCLE webserver [101] and annotated with ESPript3 [102]. A sequence similarity matrix was constructed using the SIAS (Sequence Identity and Similarity) webserver [103].

**Modeling and structure-based analysis**

Human Hydin PapD-like C-terminal ASH domain (2E6 [47]) was the best candidate template identified for modeling the SPD-2 ASH domain using various fold recognition algorithms. Three-dimensional structural models of the CeSPD-2 ASH domain were generated using template-based modeling programs as well as ab initio approaches with Modeller [104], RAPTOR-X [105], Phyre2 [106], I-TASSER [107], LOMETS [53], and Swissmodel [108]. Model quality was evaluated using ProSA-Web [109], Veriﬁy3D [110], ProQ3 [111], and VoroMQA [112]. The model with the best evaluation proﬁle was further reﬁned using ModReﬁner [113] and used for all subsequent analyses. The ab initio modeling algorithm in Robetta [114] was also used to gather predicted structural data for the disordered domains in CeSPD-2. DEMO [115] was used to assemble full-length models. Electrostatic potential maps were produced and visualized using the Adaptive Poisson-Boltzmann Solver (APBS) – PDB2PQR server [116] and the PyMOL (v.1.8) molecular graphics system [117]. Chimera (v.1.11) [118] was used for structure alignment and the production of backbone images. Potential contact sites based on the sequence and predicted structure of the model were predicted using SPPIDER (Solvent accessibility-based Protein–Protein Interface iDEntification and Recognition) [119]. The same procedure was done for modeling ASH domains of *Drosophila* abnormal spindle protein/ASP and human abnormal spindle-like microcephaly-associated protein/ASPM.

**Plasmid construct**

The pie-1P::ASH::wGFP clone was generated by synthesis (Genewiz). This construct includes the pie-1 gene promoter in the pCM1.127 plasmid (Addgene), driving a translational fusion of the SPD-2 ASH domain (nucleotides 1444–1720) and
the wGFP sequence isolated from the pCFJ1848 plasmid (optimized for germline expression [59]). The 3'UTR in this construct contains the pie-1 3'UTR from the pCM5.47 plasmid (Addgene). The hsp-16.2p::ASH::GFP construct was generated by cloning the same SPD-2 ASH domain sequence (nucleotides 1444–1720) and the wGFP sequence into the PTB11 vector (Andrew Fire plasmid kit) that includes the hsp-16.3 promoter and the unc-54 3'UTR.

Culture conditions strains

*C. elegans* hermaphrodites were cultured using standard techniques [120]. All strains were maintained at 16°C unless otherwise noted. The pie-1p::ASH::GFP plasmid was injected (15 ng/µL) into the OD95 strain – unc-119(ed3) III; lts37 IV ([pAA64] pie-1p::mCherry::his-58 + unc-119(+)); lts38 III [pie-1p::GFP::PH(PLC1delta1) + unc-119 (+)] by NemaMetrix (Murray, UT). A rol-6 construct (pNU406) was used as the injection marker. The MSC25 strain that resulted from these injections transmits the extrachromosomal to about 10% of its progeny. MSC25 roller hermaphrodites were crossed with AV675 [mCherry::H2B] males and the resulting progeny was screened for ASH::GFP expression. The hsp-16.2p::ASH::GFP plasmid (2–10ng/l) was co-injected with the rol-6 (PRF4) injection marker (15–25ng/µL) into the germline of N2 nematodes. The resulting strain (MSC26) expressed ASH::GFP upon heat shock treatment. To reduce sequence repetitiveness in extra-chromosomal arrays and prevent suppression of expression of the GFP constructs in the germline, sheered salmon sperm DNA was included to obtain a final injection mix concentration of 100 ng/µL [58].

Heat shock treatment and gonad dissection

Gravid MSC6 hermaphrodites were heat-shocked at 30°C for 4 hours. After heat-shock, nematodes were recovered at 16°C for 1–2 hours. Recovered hermaphrodites were placed in 5 µL drop of M9 on a glass slide cover slip and were dissected to release the embryos. Coverslips with dissected MSC25 or MSC26 hermaphrodites were placed onto microscope slides with a 4% agar cushion sealed with Vaseline, and embryos were immediately imaged.

Imaging

GFP fluorescence was observed in about 1 embryo out of 10 of each the strains carrying an ASH::GFP construct, because of low transmission of the extra-chromosomal array. Given the low germline transmission of the arrays in all strains most embryos show no GFP fluorescence. For each condition 100% of the same pattern ASH::GFP expression was observed. At least seven animals were observed for each expression pattern. Z-stacks images of embryos were collected with the DeltaVision Deconvolution system mounted on an Olympus IX-70 inverted microscope equipped with a CoolSnap HQ CCD camera and a LED arc bulb illumination source, using 60 × 1.514NA oil immersion objective, GFP/mCherry fluorescence filter sets, and DIC optics. Image processing and Z-stack projections were done with the SoftWorx software included in the Delta Vision Deconvolution system.

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Disclosure statement

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