The hydrolethalus syndrome protein HYLS-1 links core centriole structure to cilia formation

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Centrioles are small cylindrical organelles whose distinguishing feature is an outer wall composed of a ninefold symmetric array of stabilized microtubules. Centrioles perform two distinct functions in eukaryotic cells: (1) They build centrosomes that organize the microtubule cytoskeleton, and (2) they template cilia, microtubule-based projections with sensory and motile functions. We identified HYLS-1, a widely conserved protein, based on its direct interaction with the core centriolar protein SAS-4. HYLS-1 localization to centrioles requires SAS-4 and, like SAS-4, HYLS-1 is stably incorporated into the outer centriole wall. Unlike SAS-4, HYLS-1 is dispensable for centriole assembly and centrosome function in cell division. Instead, HYLS-1 plays an essential role in cilia formation that is conserved between Caenorhabditis elegans and vertebrates. A single amino acid change in human HYLS1 leads to a perinatal lethal disorder termed hydrolethalus syndrome, and we show that this mutation impairs HYLS-1 function in ciliogenesis. HYLS-1 is required for the apical targeting/anchoring of centrioles at the plasma membrane but not for the intraflagellar transport-dependent extension of the ciliary axoneme. These findings classify hydrolethalus syndrome as a severe human ciliopathy and shed light on the dual functionality of centrioles, defining the first stably incorporated centriolar protein that is not required for centriole assembly but instead confers on centrioles the capacity to initiate ciliogenesis.

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Centrioles are small cylindrical organelles whose distinguishing feature is an outer wall composed of a ninefold symmetric array of stabilized microtubules. Centrioles perform two distinct functions in eukaryotic cells: (1) They build centrosomes that organize the microtubule cytoskeleton, and (2) they template cilia, microtubule-based projections with sensory and motile functions. In dividing vertebrate cells, centrioles organize centrosomes and direct the formation of a primary cilium during interphase (Rieder et al. 1979). In other cell types, such as in C. elegans neurons or in multiciliated vertebrate epithelial cells, ciliogenesis is initiated following terminal differentiation in response to expression of specific transcription factors (Chen et al. 1998; Swoboda et al. 2000). Centrioles initiate ciliogenesis by translocating to
the cell surface in a step thought to involve vesicle trafficking and the actin cytoskeleton (Dawe et al. 2007; Park et al. 2008). Once anchored at the plasma membrane via a specialized structure termed the transition zone, motor-driven intraflagellar transport (IFT) extends the ciliary axoneme (Schroder 2008). The molecular mechanisms required for centrioles to translocate to the cell surface and serve as a launching pad for IFT to elongate the ciliary axoneme remain largely unknown.

Human centrioles are assembled at the plasma membrane and undergo cycles of growth and disassembly. Centrioles are critical for many cellular processes, including cell division, lipid metabolism, and cilia formation. The assembly of centrioles is a highly conserved process that involves the sequential recruitment of proteins, including SAS-4, SAS-5, SAS-6, and the kinase ZYG-1/Plk4. These proteins work together to form a cylindrical scaffold called the central pair, which is disrupted during centriole disassembly. However, the molecular mechanisms underlying these dual functions, the outer wall, composed of stabilized microtubules, mediates the acquisition of the pericentriolar matrix or directs cilia formation. To elucidate the molecular mechanisms underlying these dual functions, we performed tandem affinity purifications of GFPLAP:SAS-4 from worm extracts and identified copurifying proteins using mass spectrometry (Cheeseman and Desai 2005). This approach identified a single SAS-4-interacting protein encoded by the C05C8.9 gene as HYLS-1, a protein of unknown function mutated in the perinatal lethal disorder hydrolethalus syndrome (Mee et al. 2005). We therefore named the C05C8.9 gene hyls-1 and refer to its protein product as HYLS-1. The interaction between HYLS-1 and SAS-4 was confirmed by communoprecipitation using affinity-purified antibodies (Fig. 1C). This interaction is specific for SAS-4, as Sas-4 was not found in organisms that lack centrioles, such as fungi and higher plants. HYLS-1 is essential for IFT-dependent centriole assembly and cilia formation during cell division. HYLS-1 is specifically required for ciliogenesis.

Results

Identification of a SAS-4-associated protein that is mutated in hydrolethalus syndrome

SAS-4 directs a late step in centriole assembly, the formation of the outer centriole wall [Fig. 1A]. Depending on the differentiation/cell cycle state, the outer wall, composed of SAS-4 and the stabilized centriolar microtubules, mediates the acquisition of the pericentriolar matrix or directs cilia formation. To elucidate the molecular mechanisms underlying these dual functions, we performed tandem affinity purifications of GFPLAP:SAS-4 from worm extracts and identified copurifying proteins using mass spectrometry (Cheeseman and Desai 2005). This approach identified a single SAS-4-interacting protein encoded by the C05C8.9 predicted gene as HYLS-1. The interaction between HYLS-1 and SAS-4 was confirmed by communoprecipitation using affinity-purified antibodies (Fig. 1C). This interaction is specific for SAS-4, as Sas-4 was not found in organisms that lack centrioles, such as fungi and higher plants. HYLS-1 is essential for IFT-dependent centriole assembly and cilia formation during cell division. HYLS-1 is specifically required for ciliogenesis.

With the exception of a common contaminant (HSP-1) no other proteins were copurified in the SAS-4 and HYLS-1
tandem affinity preps, suggesting that HYLS-1 interacts directly with SAS-4. Consistent with this, a robust interaction was observed between HYLS-1 and SAS-4 in yeast two-hybrid analysis (Fig. 1E). Deletion studies identified the regions of SAS-4 and HYLS-1 responsible for the interaction (Fig. 1E). The region of HYLS-1 that
mediates the interaction with SAS-4 is distinct from the HYLS-1 box, in which the disease-linked mutation is located. Thus, hydrolethalus syndrome is not likely to be a consequence of disrupting the interaction between SAS-4 and HYLS-1.

Consistent with their biochemical association, a GFP:HYLS-1 fusion [Fig. 1F] as well as endogenous HYLS-1 [Fig. 1G,H], colocalized with SAS-4 to centrioles at the center of the pericentriolar material throughout the embryonic cell cycle as well as in mature sperm, where there is no pericentriolar material [Fig. 1F–H]. We conclude that HYLS-1, whose human ortholog is linked to hydrolethalus syndrome, directly associates with and colocalizes with the core centriolar structural protein SAS-4.

HYLS-1 is stably incorporated into newly forming centrioles in an SAS-4-dependent step

During centriole duplication in the C. elegans embryo, SAS-4 is recruited to the central tube in S phase. In prophase, SAS-4 directs assembly of the microtubules that constitute the outer centriole wall. Centriolar microtubule assembly prevents further exchange of SAS-4 with the cytoplasmic pool, irreversibly locking it into the outer wall of the new centriole (Dammermann et al. 2008). Using a previously developed method [Fig. 2A; Dammermann et al. 2008], we found that GFP:HYLS-1 was recruited to the site of new centriole assembly with timing identical to SAS-4 [Fig. 2B,C]. Depletion of SAS-4 by RNAi abolished centriolar recruitment of HYLS-1, but not vice versa, placing HYLS-1 below SAS-4 in the centriole assembly pathway [Fig. 2B,C]. Photobleaching experiments revealed that centriolar HYLS-1, like SAS-4, ceases to exchange with the cytoplasmic pool during late prophase [Fig. 2D,E], indicating that HYLS-1 is stably incorporated into the new centriole along with SAS-4 when the outer wall microtubules assemble. These in vivo results validate the interaction between SAS-4 and HYLS-1 observed using biochemistry and two-hybrid analysis and indicate that HYLS-1, like SAS-4, is stably incorporated into centrioles during their assembly, beginning with the first new centrioles formed after fertilization in the one-cell embryo.

HYLS-1 is dispensable for centriole duplication, centrosome function in cell division, and embryonic viability

SAS-4 is required for the formation of the microtubule-containing outer centriole wall. Following its depletion in the early embryo, the two sperm-derived centrioles separate but fail to duplicate (Kirkham et al. 2003). Consequently, each daughter cell inherits a single centriole, leading to monopolar spindle formation in the second mitotic division and penetrant embryonic lethality. Surprisingly, despite the direct association of HYLS-1 with SAS-4 and their similar behavior during centriole assembly, HYLS-1-depleted embryos exhibited no defects in centriole duplication and no significant increase in embryonic lethality [Fig. 3A]. To confirm this unexpected finding, we obtained a deletion mutant, hyls-1(tm3067) [Supplemental Fig. S1A], hereafter referred to as hyls-1Δ, which has no detectable HYLS-1 expression by immunoblot [Fig. 3B] or immunofluorescence (Supplemental Fig. S1C). In agreement with the RNAi results, hyls-1Δ embryos did not show centriole duplication defects, were viable, and developed into fertile adults [Fig. 3A]. Thus, even though HYLS-1 is associated with SAS-4 and stably incorporated into centrioles during their assembly, it is dispensable for centriole duplication and centrosome function during cell division.

HYLS-1 is required for ciliogenesis

In addition to organizing centrosomes, centrioles serve as basal bodies that template cilia. To determine whether HYLS-1 contributes to cilia formation, we monitored ciliogenesis in hyls-1Δ animals using a dye-fill assay. Of the 60 ciliated neurons in C. elegans hermaphrodites, 12 amphid [head] and four phasmid [tail] neurons take up the lipophilic dye DiI through their exposed ciliated endings and accumulate it in their cell bodies (Supplemental Fig. S2A–C; Hedgecock et al. 1985). In hyls-1Δ animals, only ~10% of the normal number of ciliated neurons were observed to dye-fill [Fig. 3C; Supplemental Fig. S2D]. Neuron-specific GFP reporters confirmed normal presence of the amphid and phasmid neurons [Figs. 3D, S2A,B], indicating that the dye-filling defect is not due to absence of the neurons themselves. Albeit infrequent, the low level of dye-filling in hyls-1Δ animals is in contrast to IFT-defective mutants, which completely lack dye-filling neurons. Placing the hyls-1Δ mutant allele over a deficiency did not reduce the number of residual cilia [1.2 ± 0.9 dyf(+) neurons in hyls-1Δ/Δ; 1.6 ± 1.4 hyls-1Δ/Δ deficiency; n = 35 animals per condition], excluding the possibility that the deletion mutant is hypomorphic. Residual cilia were not associated with a particular type of neuron and the dye-fill pattern was not heritable [data not shown]. These results establish that HYLS-1 plays an important role in cilia assembly in C. elegans.

Neuronal expression of HYLS-1 is not sufficient for ciliogenesis

The requirement for HYLS-1 in ciliogenesis but not cell division raises the question as to why HYLS-1 is incorporated into all centrioles, starting with those assembled in the first embryonic division. One possibility is that HYLS-1 must be present on centrioles to perform its function in initiating ciliogenesis. Since centrioles are distributed randomly to daughter cells in C. elegans embryos (our unpublished results), the assembly-coupled incorporation of HYLS-1 into all centrioles would guarantee its presence in the centrioles inherited by ciliated neurons. To test this idea, we generated strains carrying integrated transgenes directing expression of GFP:HYLS-1 under control of the pie-1 promotor, which directs expression in early embryos, and the rgef-1 promotor, which directs expression in all post-mitotic neurons (Altun-Gultekin et al. 2001), and compared their ability to rescue ciliogenesis in the hyls-1Δ mutant. Expression of GFP:HYLS-1 under the control of the pie-1 promotor
fully rescued the dye-filling defect in the hyls-1 D mutant (Fig. 3C, Supplemental Fig. S2D). By contrast, expression under the control of the rgef-1 promoter failed to rescue ciliogenesis in the majority of neurons (Fig. 3C; Supplemental Fig. S2D). These results suggest that the assembly-coupled incorporation of HYLS-1 into centrioles inherited by ciliated neurons is necessary for it to perform its role in ciliogenesis.

HYLS-1 is present at the base of mature cilia

To examine if HYLS-1 protein is present in mature cilia, we performed immunofluorescence of late (threefold stage) embryos and early (L1) larvae with antibodies to HYLS-1. HYLS-1 localized to punctate structures in amphid and labial/cephalic neurons (Fig. 4A–D). This punctate staining was absent in hyls-1 Δ worms and was fully restored.

Figure 2. HYLS-1 is stably incorporated into newly forming centrioles in an SAS-4-dependent manner. (A) Schematic of the method used to analyze recruitment of centriolar proteins to site of centriole assembly (Dammermann et al. 2008). Briefly, sperm centrioles labeled with mCherry:SAS-4 were introduced by mating into oocytes expressing a GFP fusion with HYLS-1 or SAS-4. Recruitment of the GFP-labeled protein to newly formed centrioles was quantified by measuring the GFP fluorescence coincident with the mCherry signal at each time point. Measurements from multiple embryos were pooled and averaged to generate each recruitment plot. (B, C) Representative images (B) and quantification (C) of centriolar GFP:SAS-4 or GFP:HYLS-1 recruitment in control embryos and embryos depleted of HYLS-1 or SAS-4 as indicated. HYLS-1 recruitment requires SAS-4 but not vice versa. Times are in seconds relative to cytokinesis onset. Error bars are the 90% confidence interval. (D) SAS-4 is recruited to centrioles during the formation of the central tube in S phase, but remains in dynamic exchange with the cytoplasmic pool of SAS-4 until assembly of the centriolar microtubules in late prophase locks it into the outer centriole wall (Dammermann et al. 2008). (E) GFP:SAS-4 and GFP:HYLS-1 exhibit an identical behavior in a photobleaching-based assay assessing the ability of the centriolar protein to exchange with cytoplasmic pools. Recovery is observed if centrioles are bleached in early prophase (GFP:SAS-4, n = 8 out of 8; GFP:HYLS-1, n = 8 out of 8), but not if centrioles are bleached in late prophase (GFP:SAS-4, n = 1 out of 22; GFP:HYLS-1, n = 0 out of 9). Bars, 2 μm.
by pie-1 promoter-regulated HYLS-1 expression in the deletion mutant; by contrast, rgef-1 promoter-regulated neuronal expression of HYLS-1 in the deletion mutant resulted in a few puncta but failed to restore the wild-type pattern (Fig. 4C).

To determine whether the HYLS-1 puncta are at the base of the cilia, we examined the localization of HYLS-1 relative to NPH-4:YFP, a marker for the ciliary transition zone [Williams et al. 2008]. HYLS-1 puncta were closely apposed to the short stretches of NPH-4:YFP [Fig. 4A], indicating that HYLS-1 localizes to a discrete structure at the base of cilia. We confirmed this result using CHE-GFP, an IFT component that localizes to the transition zone and along the ciliary axoneme [Ou et al. 2005]. Localization to the base of the cillum was also observed in phasmid neurons [Supplemental Fig. S2E]. We conclude that HYLS-1 localizes to a discrete structure at the base of the mature cillum, consistent with its function in ciliogenesis.

HYLS-1 localization in ciliated neurons is independent of the master ciliogenesis regulator DAF-19

In C. elegans, ciliogenesis occurs during terminal differentiation in response to expression of the DAF-19 transcription factor, whose targets include the IFT machinery as well as the Bardet-Biedl and Meckel syndrome proteins [Swoboda et al. 2000]. In daf-19(m86) mutants, the ciliogenesis program is not activated and cilia assembly fails [Perkins et al. 1986]. However, HYLS-1 localization was unaffected [Fig. 4C]. This finding is consistent with the observations that HYLS-1 is incorporated into all centrioles during their assembly and that expression of HYLS-1 from a pan-neuronal promoter fails to restore ciliogenesis in hyls-1D animals.

C. elegans is unusual in that mature cilia do not terminate in centrioles/basal bodies recognizable by electron microscopy. Instead, structures similar to the “transition zone” found above the basal body in other organisms anchor cilia to the plasma membrane [Inglis et al. 2007]. As the initiation and early steps of ciliogenesis, which occur in the late embryo where cells are small and cell density is very high, have not been visualized using either light or electron microscopy, it is unclear when centriolar structure is lost during differentiation. Immunofluorescence of late embryos with antibodies to HYLS-1 and SAS-4 revealed puncta containing both SAS-4 and HYLS-1 throughout the body of the animal, likely corresponding to centrioles. By contrast, in the ciliated neurons of the
head, puncta containing HYLS-1, but not SAS-4, were observed [Fig. 4D]. Thus, in C. elegans, two interacting outer centriole wall components are uncoupled in mature cilia, with HYLS-1, but not SAS-4, present at the ciliary base. Elucidation of the timing of this uncoupling and the mechanisms underlying loss of centriolar structure will require methods to visualize the early steps of ciliogenesis. We conclude that in C. elegans HYLS-1 localization to the ciliary base is independent of the master ciliogenesis regulator DAF-19 and persists after loss of centriole structure in mature cilia.

HYLS-1 acts in a step functionally distinct from IFT

To determine how HYLS-1 contributes to ciliogenesis, we analyzed a GFP fusion with the IFT component CHE-11 [Fig. 5A; Supplemental Movies S1, S2; Ou et al. 2005]. In the 10% residual cilia in hyls-1Δ animals, anterograde movement of CHE-11:GFP particles occurred at a similar rate to wild-type, indicating that HYLS-1 is dispensable for IFT [Fig. 5A]. In wild-type and IFT-defective osm-6(p811) animals, CHE-11:GFP accumulated at a focus, which is the transition zone at the base of the mature C. elegans cilium in the wild-type. By contrast, in hyls-1Δ animals, no CHE-11:GFP accumulation was seen in the majority of neurons [Fig. 5B]. Thus, HYLS-1 is required to form a normal transition zone, but not for the IFT-dependent extension of the ciliary axoneme.

Ciliogenesis and cilia-dependent behaviors are compromised by the disease-associated missense mutation in the HYLS-1 box

Cilia, while dispensable for viability and fertility in C. elegans, are required for a variety of behaviors including chemotaxis, foraging, and male mating. Consistent with their severe cilia assembly defect, the performance of hyls-1Δ animals in behavioral assays was similar to that of other ciliary mutants [Fig. 5C–E]. Expression of a GFP fusion with wild-type HYLS-1 rescued the dye-fill

Figure 4. HYLS-1 is present at the base of mature C. elegans cilia. (A) HYLS-1 localizes to the base of the cilium. Late (threefold)-stage embryo expressing the transition zone marker NPH-4:YFP stained for DNA, tubulin, and HYLS-1. The inset shows a magnified view of amphid bundle (arrowhead). (B) L1 larva expressing the IFT protein CHE-11:GFP stained for DNA, tubulin, and HYLS-1. (C) Expression of wild-type or disease mutant HYLS-1 from a germline promoter (Ppie-1) restores localization in hyls-1Δ animals, whereas expression from a pan-neuronal promoter (Prgef-1) fails to do so. Late-stage embryos stained for DNA, tubulin, and HYLS-1. (D) SAS-4 is not present at the ciliary base (panel i), although it colocalizes with HYLS-1 at centrioles elsewhere in the animal (panel ii). Late-stage embryo stained for DNA, tubulin, HYLS-1, and SAS-4. Bars: A–C, 5 μm; D, top panel, 50 μm; D, panels i, ii, 10 μm.
defect of \textit{hyls-1}\textDelta worms as well as all three analyzed cilia-dependent behaviors. To examine the functional consequences of the hydrothelatus syndrome-associated mutation, we generated a GFP fusion with HYLS-1 containing the D210G mutation in the HYLS-1 box. Mutant and wild-type proteins were expressed at similar levels (Supplemental Fig. S1D), and both localized to centrioles (Supplemental Fig. S1C) and the base of cilia (Fig. 4C), consistent with the finding that the region of HYLS-1 that interacts with SAS-4 is distinct from the HYLS-1 box (Fig. 1E). However, mutant HYLS-1 was compromised in its ability to completely restore cilia assembly in the \textit{hyls-1}\textDelta mutant, explaining the difference in the number of particles. 

\begin{figure}
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\includegraphics[width=\textwidth]{image.png}
\caption{HYLS-1 acts in an IFT-independent early step in ciliogenesis. (A) IFT is unchanged in residual cilia of \textit{hyls-1}\textDelta animals. Stills from time-lapse sequences of amphid neurons expressing CHE-11:GFP [Supplemental Movies S1, S2] and kymographs showing IFT particle movement along ciliary axonemes. IFT rates were determined for particles in the distal segment. Note that there are eight ciliated neurons in each amphid bundle in wild-type, but only a single cilium in the \textit{hyls-1}\textDelta mutant, explaining the difference in the number of particles. (B) CHE-11:GFP fails to accumulate at the transition zone of \textit{hyls-1}\textDelta animals. Stills from time-lapse sequences of phasmid neurons expressing CHE-11:GFP. Arrowheads indicate position of transition zone. (C) Results of chemotaxis assays performed with wild-type and mutant animals. Each data point represents one assay with \textgreater{}100 animals. (D) Male mating efficiency of wild-type and mutant animals. The ability of individual L4 males to produce cross-progeny with \textit{unc-119} mutant hermaphrodites was scored (percentage of males producing cross-progeny; \textit{n} = 20 per genotype). (E) Foraging behavior of wild-type and mutant animals. Tracks generated by a single animal left for \textit{16 h} on an NGM plate seeded with \textit{OP50} bacteria are shown. While wild-type animals efficiently explore their environment, cilia-defective mutant animals fail to do so, exhibiting a dwelling phenotype [Fujiwara et al. 2002]. Bars: A, B, \textit{5 \mu m}; E, \textit{1 cm}.
\end{figure}

The role of HYLS1 in ciliogenesis is conserved in vertebrates

To determine if the function of HYLS-1 in ciliogenesis is conserved in vertebrates, we turned to \textit{Xenopus laevis} as an experimental model. The epidermis of amphibian larvae is a mucociliary epithelium, with multiciliated cells interspersed among nonciliated mucus-secreting cells. Antisense morpholino oligonucleotides, injected on their own or with morpholino-resistant mRNAs, can be used to examine the requirement of specific factors for cilia assembly or function (Fig. 6A; Mitchell et al. 2007; Park et al. 2008).

Morpholino-based depletion of XHYLS-1 resulted in a specific loss of cilia, with no effect on the number of cells with multiple basal bodies or the number of basal bodies per cell (Fig. 6B,D). Thus, as in \textit{C. elegans}, \textit{Xenopus} HYLS-1 is dispensable for centriole assembly and cell division, but is specifically required for cilia formation. GFP fusions with both wild-type and disease mutant XHYLS-1, expressed using mRNA injections into two-cell embryos, localized to basal bodies in multiciliated cells [Fig. 6C]; however, only the wild-type GFP:XHYLS-1 restored ciliogenesis in embryos depleted of endogenous XHYLS-1 [Fig. 6C,D]. Thus, HYLS-1 is required for ciliogenesis in vertebrate multiciliated epithelia, indicating that its function is conserved between \textit{C. elegans} and vertebrates. In addition, the disease-associated mutation has a more severe effect on cilia assembly in multiciliated epithelia, which may reflect a species difference and/or the increased burden associated with building \textit{\textgreater{}150} cilia per cell.
HYLS1 is required for the apical organization of basal bodies

In epithelia, cilia extend from basal bodies that are evenly distributed over the apical cell surface. In HYLS-1 depletions, basal bodies were clustered and spatially disorganized within the cell, with the most noticeable defect being a lack of apical localization. This defect was most clearly evident in x-z projections of three-dimensional (3D) image stacks (Fig. 7A,B). Apical localization was restored upon expression of wild-type but not disease mutant (D249G) XHYLS-1 fused to GFP restores ciliogenesis in XHYLS-1-depleted embryos. (D) Quantification of morpholino experiments. n = 10–12 embryos, 30 fields of view per embryo [cells with multiple cilia/basal bodies]; 20 cells [basal bodies per cell]. Bars, 10 μm. Error bars in D are the 95% confidence interval.

Discussion

HYLS1 acts as a centriolar scaffold for cilia assembly

It has long been appreciated that a primary role of centrioles, conserved in all species that contain them, is to generate cilia. Our analysis indicates that HYLS-1 is a core centriolar protein that is stably incorporated into the outer wall during centriole assembly specifically to confer on centrioles the capacity to initiate ciliogenesis (Fig. 7C). A defect in this capacity leads to a lethal human developmental disorder, hydrolethalus syndrome. HYLS-1 is conserved in evolution from ciliates to vertebrates, indicating that ciliogenesis in all eukaryotes is likely to require a centriole-intrinsic mechanism involving HYLS-1.

Hydrolethalus syndrome is a cilia-related disorder

The single amino acid substitution in HYLS1 linked to hydrolethalus syndrome impairs its function in ciliogenesis, indicating that this developmental disorder is a previously unrecognized ciliopathy. Despite the severity
of hydrolethalus syndrome, our results suggest that the hydrolethalus-associated missense mutation, which changes an invariant aspartic acid in the conserved HYLS-1 box to glycine, is not a null mutation. HYLS-1 containing the hydrolethalus mutation is stable, associates with SAS-4, and targets to centrioles/basal bodies in both *C. elegans* and *Xenopus*. In the sensory (primary) cilia of *C. elegans*, the hydrolethalus mutation only mildly compromised rescue of the ciliary dye-fill phenotype of the *hyls-1* deletion, although complex cilia-dependent behaviors were significantly perturbed. In contrast, in the multiciliated epithelium of *Xenopus* embryos, the mutant protein exhibited little ability to rescue cilia formation. The difference between the consequences of the disease-associated mutation in *C. elegans* and *Xenopus* may reflect either a species difference, the nature of the cilia assayed (primary sensory cilia in *C. elegans*, motile cilia in *Xenopus*), or the two orders of magnitude difference in the number of cilia assembled per cell (one in *C. elegans* neurons; ~150 per ciliated epithelial cell). While future work is needed to discriminate between these possibilities, the strong defect associated with the disease mutation in multiciliated vertebrate epithelia may account for the severity of hydrolethalus syndrome.

The clinical features of hydrolethalus syndrome are most similar to those of Meckel-Gruber syndrome (Salonen and Herva 1990). Both are characterized by a majority of affected individuals dying in utero, CNS malformations, and polydactyly, although hydrolethalus syndrome does not share the cystic dysplasia of the kidneys or fibrous

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**Figure 7.** XHYLS-1 is required for apical targeting/anchoring of basal bodies. (A) Representative images of control embryos and embryos depleted of XHYLS-1, Polaris or IFT-52, stained with antibodies to acetylated tubulin and γ-tubulin to illustrate effect on basal body distribution. Images are x–y projections of deconvolved 3D data stacks. (B) x–z projection of 3D data stacks presented in A and Figure 6C. (C) Model for the role of HYLS-1 in cilia formation. HYLS-1 is recruited to the forming centriole by its association with SAS-4, and like SAS-4 is locked into the centriole during the assembly of centriolar microtubules. Incorporation of HYLS-1 confers on centrioles the capacity to initiate ciliogenesis. Bars, 10 μm.
changes of the liver associated with Meckel-Gruber syndrome. Meckel-Gruber syndrome is related to two less severe ciliopathies, nephronophthisis and Joubert syndrome, in that these three phenotypically distinct disorders are linked to different lesions in the same genetic loci. These loci include MKS3/JBTS6 [Baala et al. 2007b], NPHP1/JBTS4 [Parisi et al. 2004], MKS4/NPHP6/JBTS5 [Sayer et al. 2006, Valente et al. 2006; Baala et al. 2007a], and MKS5/NPHP8/JBTS7 [Arts et al. 2007, Delous et al. 2007]. Notably, the genes currently identified for Meckel-Gruber syndrome, nephronophthisis, and Joubert syndrome are only mutated in a small percentage of affected individuals, suggesting that other loci responsible for these disorders remain to be identified [Khaddour et al. 2007; Hildebrandt et al. 2009]. It is possible that mutations in HYLS1, distinct from the one linked to hydrothalmus syndrome, are associated with these or other ciliary disorders. Future efforts to sequence the HYLS1 locus in affected individuals should help address this possibility.

HYLS-1 acts in an early step in the ciliogenesis pathway

The apical migration and anchoring of centrioles at the cell surface is a critical initial step in ciliogenesis. Whether the centriole plays an active role in apical migration and anchoring has until now been unclear; our results suggest that HYLS-1 provides a centriole-intrinsic mechanism that interfaces with other processes to organize basal bodies at the cell surface and support elongation of the ciliary axoneme. Perturbations of apico–basal polarity and the cortical actin cytoskeleton have been shown to disrupt apical targeting of basal bodies [Boisvieux-Ulrich et al. 1990; Pan et al. 2007; Park et al. 2008]. Early EM studies also suggested potential involvement of vesicle trafficking [Sorokin 1962] and components of the exoyct, a complex involved in tethering vesicles to the plasma membrane, have been localized recently to basal bodies [Park et al. 2008; Zuo et al. 2009].

Elucidating the molecular mechanism by which HYLS-1 contributes to basal body organization at the cell surface and helps initiate cilia formation will require identification of its effectors. The proteins mutated in Meckel syndrome, Joubert syndrome, and Nephronophthisis are good candidates for downstream effectors of HYLS-1 in the ciliogenesis pathway. Homologs of three of the gene products linked to Meckel-Gruber syndrome, Joubert syndrome, and Nephronophthisis in humans, MKS-1, NPH-1 and NPH-4, have been characterized in C. elegans. MKS-1 contains a B9 domain that is found in two other proteins, called TZA-1 and TZA-2. All three B9 domain proteins as well as the nephrocystins NPH-1 and NPH-4 localize to the transition zone in ciliated neurons. Unlike HYLS-1, expression of the nephrocystins, MKS-1, TZA-1, and TZA-2 is under control of DAF-19 [Winkelbauer et al. 2005; Williams et al. 2008], supporting the idea that HYLS-1 acts upstream of these components. While individual inhibition of MKS-1, TZA-1, TZA-2, NPH-1, or NPH-4 does not result in a significant defect in cilia morphology [Jauregui and Barr 2005; Winkelbauer et al. 2005; Jauregui et al. 2008, Williams et al. 2008; Bialas et al. 2009], simultaneous inhibition of a B9 domain-containing protein and either NPH-1 or NPH-4 results in a dye-filling defect reminiscent of HYLS-1 inhibition [Williams et al. 2008]. The reasons for the functional redundancy between these components are currently unclear. Future work is needed to understand the basis for this redundancy and to determine whether there are functional interactions between these ciliogenesis-specific proteins and HYLS-1 in the early stages of the ciliogenesis pathway.

Materials and methods

LAP purifications, immunoprecipitations, and yeast two-hybrid

LAP preps were performed on high-speed supernatants from whole-worm extracts as described [Cheeseman and Desai 2005] and interacting proteins were identified by LC/MS/MS. Immunoprecipitations were performed on embryo extracts as described previously [Desai et al. 2003]. Yeast two-hybrid experiments were performed using the Matchmaker two-hybrid system (Clontech).

Sequence analysis

HYLS-1 orthologs were identified by PSI-BLAST using the conserved sequence shown in Figure 1D [which extends from amino acid 205 to 246 in human HYLS-1] as query. The Xenopus ortholog was cloned by PCR from cDNA prepared from tailbud-stage embryos. Sequence alignments were performed with MUSCLE in Jalview [http://www.jalview.org]. Accession numbers are as follows: Hs (Homo sapiens) NP_659451; Mm [Mus musculus] AAH35953; XI (X. laevis) UniGene XL.13199; Gg [Gallus gallus] XP_001231543; Dm (Drosophila melanogaster) CG42231 (FlyBase); Ce (C. elegans) NP_504840; Mb (Monosiga brevicollis) EDQ89544; Nv (Nematostella vectensis) XP_001640703; Th (Trypanosoma brucei) XP_803779; Lm (Leishmania major) XP_001687472; Pt (Paramecium tetraurelia) XP_001437786.

Materials and methods

C. elegans experiments

Worm strains are listed in Supplemental Table S1. The hyls-1 deletion mutant obtained from the C. elegans knockout consortium (NBP-Japan) was outcrossed six times against N2 wild-type before phenotypic analysis and introduction of GFP markers. Strains were maintained at 16°C (OD19 and OD113) or 22°C (all others). RNAi experiments were performed as described previously [Dammermann et al. 2008] using the dsRNAs listed in Supplemental Table S2. Recruitment curves, high-resolution imaging, and FRAP analyses were performed as described previously [Dammermann et al. 2008]. Immunofluorescence experiments on early and late embryos were performed using directly labeled affinity-purified antibodies to α-tubulin [DM1a-FITC, Sigma], SAS-4 [Dammermann et al. 2004], and HYLS-1 [full-length] [this study] as described previously [Oegema et al. 2001]. Immunoblots were performed on whole-worm extracts with affinity-purified antibodies to full-length HYLS-1 or GFP, using anti-α-tubulin as a loading control [DM1a, Sigma].

C. elegans ciliary assays

Dye-fill assays were carried out by incubating ~100 worms in a 1/500 dilution of Dil [invitrogen] in M9 buffer for 1 h, allowing worms to destain on a seeded NGM plate for ~30 min before
Cilia assembly requires HYLS-1

Xenopus experiments

Xenopus embryos were obtained by in vitro fertilization according to standard methods and staged as described (Nieuwkoop and Faber 1967). Morpholino antisense oligonucleotides (GeneTools) were designed to inhibit expression of XHYLS-1, IFT52, and Polaris by targeting the initiation codon of the target mRNA. In the case of XHYLS-1, two morpholinos were necessary to target the two forms encoded in the pseudotetraploid Xenopus genome. In the case of IFT52 and Polaris, one morpholino was sufficient. The sequences were as follows: control, 5′-GGATGCTGAGGAGTCTGAGGGCTGCTGAGCTG-3′; XHYLS-1.1, 5′-GAACCTGCTGCTGTCTCAGGACACATG-3′; XHYLS-1.2, 5′-GAACCTGCTGCTGTCTCAGGACACATG-3′; IFT52, 5′-AAAGAATCTTTTGTGACTCCAT-3′; Polaris, 5′-GCCAGAGTGGACATTTGGCATC-3′.

For expression of GFP:XHYLS-1, full-length XHYLS-1 coding sequence was inserted into pCS2+ and fused to GFP at the N terminus. A mutant form corresponding to the single amino acid substitution found in hydrolethalus syndrome (D249G) was inserted into pCS2 template by in vitro transcription using SP6 (Promega). RNA was prepared from the genome.

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imaging using a 20× 0.75 NA PlanApochromat lens on a Nikon E800 upright microscope equipped with a Hamamatsu Orca ER CCD camera. To monitor IFT and individual neurons, worms were anesthetized with 10 mM levamisole, mounted on agarose pads and imaged using a 100×, 1.4 NA PlanApochromat lens and 1.5× optivar on a Nikon TE2000-E inverted microscope equipped with a krypton–argon 2.5-W water-cooled laser (Spectra-Physics) and an iXon electron multiplication back-thinned CCD camera (Andor Technology). Kymographs and movies were generated using Metamorph software. Chemotaxis assays were performed as described previously (Bargmann et al. 1993). To assay male mating efficiency of cilia mutant strains, males were generated using him-8 RNAi to induce X-chromosome misse-

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