Nuclear roles for cilia-associated proteins

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Abstract: Cilia appear to be derived, evolutionarily, from structures present in the ancestral (pre-ciliary) eukaryote, such as microtubule-based vesicle trafficking and chromosome segregation systems. Experimental observations suggest that the ciliary targeting complex, which mediates the selective movement between cytoplasmic and ciliary compartments, shares features with nuclear pores [see 1]. Our hypothesis is that this shared transport machinery is responsible for observation that a number of ciliary and ciliogenesis associated proteins are found within nuclei, where they play roles in the regulation of gene expression, DNA repair, and nuclear import and export. Recognizing the potential for such nuclear roles is critical when considering the phenotypic effects that arise from the mutational modification of ciliary proteins.

Introduction: Cilia, whether immotile primary cilia, motile cilia, flagella (on sperm), or the cilia-based structures within photoreceptor cells, represent a distinct cytoplasmic and plasma membrane domain [2]. As has been reviewed recently, mutations in cilia- or ciliogenesis associated proteins (CAPs)[1] can lead to a range of human pathologies, known collectively as ciliopathies [2-11]. In addition to effects on fluid flow and cellular swimming, cilia have been linked to Hedgehog, TGF-β, Wnt, PDGFRα, Notch, and Hippo inter-cellular signaling as well as DNA damage repair systems [5, 7, 12]. The outcomes of disrupting ciliary assembly and function therefore depend on biological context. In the zebrafish, Huang & Schier [13] found that genetic abolition of maternal and zygotic cilia formation left Wnt signaling intact while dysregulating Hh signaling.

Ciliopathic effects may also arise via altered CAP function occurring elsewhere in the cell. Dysfunction of nuclear roles for CAPs may lead to subtle changes in cellular processes

[1] Abbreviation used: ciliary and ciliogenesis associated proteins (CAPs)

such as cell cycle length, asymmetric inheritance of cellular components upon cytokinesis, and mitotic spindle orientation that can alter the fate decisions of actively proliferating neural progenitor cells (see [14], leading to defects in early CNS development that are revealed postnatally. Here we consider the possibility the nuclear functions of CAPs, arising in part from their evolutionary ancestry, can contribute to ciliopathy phenotypes in through both developmentally specific and more generic mechanisms.

Implications of the evolutionary origin of cilia: While speculating about processes that occurred billions of years ago can be problematic, there is a clear consensus that the evolution of cilia began in an ancestor that contained a nucleus, with nuclear pores mediating molecular movement across the nuclear envelope membranes and microtubule-based systems involved in chromosome segregation [15-17]. Entry into the ciliary domain is regulated through a distinct "transition zone" between non-ciliary cytoplasm and plasma membrane regions. The observation that nuclear transport proteins and nuclear localization-like sequences are involved in ciliary transport [1, 18, 19] and blocking nuclear transport also blocks ciliary import [20, 21], suggests that nuclear pore / transport proteins were co-opted during ciliary evolution. There is therefore a general possibility that a significant subset of CAPs contain ciliary-/ nuclear-localization sequences that either have yet to be removed or are functionally important. As examples, both the ciliary axoneme-associated radial spoke protein 3 (RSPH3 – OMIM: 615876)[22] and pericentriolar and ciliary basal body associated protein pericentrin (PCNT: OMIM: 605925) [23] have been found to contain both nuclear localization (NLS) and nuclear exclusion (NES) sequences. Similarly parafusin, a signaling scaffolding protein, has been found localized to the base of primary cilia in a variety of mammalian cell types and within the nuclei of fibroblasts [24]. The question arises, how common is it to find CAPs in nuclei and what roles, if any, do they play there?

Characterizing cilia-associated proteins (CAPs): Eukaryotic cilia are complex organelles composed of modified (double and triplet) microtubules and various associated proteins. Ishikawa et al [25] identified 195 polypeptides in primary cilia, of which ~75% appeared to be present in motile cilia as well. Proximity labeling studies [26] have identified over 370 cilia-associated proteins (CAPs).[2] Boldt et al [27] used an affinity proteomic-based study to identify

1319 cilia-associated proteins, 4905 inter-polypeptide interactions, and 52 molecular complexes. Such interaction complexity highlights the heterogeneous nature of ciliary functions in specific cell types and the likelihood that multiple evolutionary events along the vertebrate lineage have led to multiple functions for many of these proteins.

That said, just because a protein is found associated with other ciliary components does not insure that its primary or sole function is ciliary. Many proteins are poorly studied, apparently for largely historical rather that functional criteria; Pandey et al [28] coined the term ignorome for such proteins (genes). A dramatic example of the size and significance of the ignorome is supplied by the studies of Hutchinson et al [29] who produced a minimal bacterial genome (JCV-syn3.0) containing 473 genes. Analysis of JCV-syn3.0 revealed an ignorome of 149 genes of unknown function (~32% of the total genome); these are genes required to produce a viable organism. At the same time there is an increasing awareness that a number of proteins, originally identified as structural or functional components of one cellular system can have quite distinct functions in another; they are what we might term “multi-taskers”. One example of such multitasking proteins are the catenins. Originally identified in the context of cadherin-based cell adhesion junctions [30], catenins were subsequently recognized to play important roles in the extracellular signaling mediated regulation of gene expression [31, 32]. In this light, Kustatscher and Rappsilber suggested that proteins have a certain degree of “fuzzy” distribution within cells [33]. This notion, strengthened by proteomic analysis of subcellular compartments and organelle fractionation, indicates that the stochastic intracellular movements is evolutionarily advantageous, as it increases the interaction potential of multitasker proteins. Contextually advantageous interactions are eventually “captured” by positive selection. This may well be the case for ciliary proteins in the nucleus, where their shared functions within related structures put them in position to acquire novel roles under specific circumstances.

One approach to defining the extent to which cilia-associated proteins are localized to nuclei involves mass spectrometric-proteomic studies of isolated nuclei. There are often technical obstacles to overcome in such an analysis, including the possibility of protein leakage and redistribution during nuclear isolation [see 34]. In their study Wuhr et al [35] exploited unique aspects of the *Xenopus laevis* oocyte nucleus (the germinal vesicle), specifically its large size (~400 μm in diameter) and the ability to isolate intact germinal vesicles rapidly from

late stage oocytes (**FIG. 1**)[36]. At the same time, oocytes are not known to express cilia of any kind, which limits the analysis to those proteins expressed during later stages of oogenesis. Wuhr et al characterized proteins based on their "relative nuclear concentration (RNC), defined as the ratio of concentrations in the nucleus to the concentrations in nucleus plus the cytoplasm"; the RNC ranges from 1 for a totally nuclear protein to 0 for a completely cytoplasmic (non-nuclear) protein. Of course polypeptides that reside in the nucleus are synthesized in the cytoplasm, nuclear isolation is not completely "clean", and a number of proteins are known to shuttle between nuclear and cytoplasmic compartments, so most RNC values are intermediate between 0 and 1. Nevertheless, it is possible to distinguish classes of proteins that appear to be excluded from nuclei and confined to the cytoplasm (value of RNC < 0.1) from those that have a significant nuclear localization signal (RNC > 0.35).

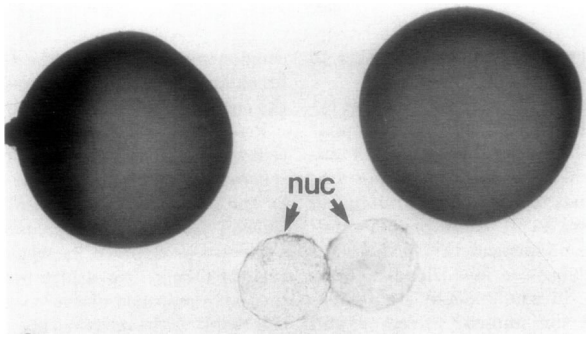

FIGURE 1: Isolated Xenopus germinal vesicles (image from Klymkowsky & Maynell, 1989); each oocyte has a diameter of ~1 mm.

To estimate the number of CAPs localized to nuclei we used the Wuhr et al. dataset together with the "gold standard" set of ~300 cilia-associated polypeptides described by van Dam et al [37][3]; to this database we added polypeptides known to be involve in ciliary function, i.e., C2orf59 [38], Cetn2 [39-41], and EFHC1 and EFHC2 [see 42], for a total of 307 polypeptides (see supplementary pdf). Of the 307 "gold standard" ciliary proteins, Wuhr et al report data on 118; of these 30 (~25%) have RNC values of > 0.35 (**TABLE 1**) and so are expected to have a significant nuclear presence. Another 29 polypeptides (~24%) have RNC values between 0.35 and 0.1) and may well be able to enter nuclei. Within subgroups of related proteins, such as the interflagellar transport polypeptides (IFTs), most appear exclusively cytoplasmic, but a few (e.g. IFT27)(**TABLE 1**) seem to be present at significant levels within the germinal vesicle. Similarly, the radial spoke protein RSPH4A (OMIM: 612647) has an RNC value of 0.9, higher than the known transcription factor SOX13 (0.84), suggesting that RSPH4A is strongly concentrated in the germinal vesicle. In contrast β-catenin (CTNNB1), which is well known to be able to enter nuclei in response to canonical Wnt

[3] Website accessible here: http://www.syscilia.org/goldstandard.shtml

signaling [see 32], appears to be exclusively cytoplasmic (RNC = 0.031) in the oocyte. In a related set of studies “nuclear” fractions were prepared from human SH-SY5Y neuroblastoma cells and analyzed by mass spectrometry (Old et al., in preparation); of the 307 “gold standard” ciliary proteins 93 were found within this nuclear fraction, including 15 that also had a “nuclear presence” in the *Xenopus* germinal vesicle data set (supplementary data 2). Together these data support the hypothesis that a significant percentage of the polypeptides linked to cilia formation, function, and phenotypic defects appear to be able to enter, and potentially play functional roles, in the nucleus. At this point we describe a few of the best characterized of these nuclear functions.

Examples of CAPs with nuclear roles: Nephronophthisis (NPH) is an autosomal recessive cystic kidney disease characterized by inflammation and scarring (fibrosis) that compromises kidney function and leads over time to end-stage renal disease [43]. Many of the genes linked to NPH, encoding polypeptides known as **nephrocystins** or NPHPs, have been found to encode proteins localized to or involved in primary cilia formation or cilia dependent signaling (both Wnt and Shh) which presumably explains phenotypic effects on other organ systems; where other organs are involved the disease is termed NPH-related ciliopathy [43]. Of the NPHP-associated genes, a number have been found to have direct or indirect nuclear roles. For example, **NPHP4** (nephrocystin-2: OMIM 607215) interacts with and inhibits the LATS1 kinase, leading to the nuclear accumulation of the transcriptional co-activators YAP and TAZ, two proteins involved in the HIPPO (hypoxia) signaling system [44]. **NPHP9** (also known as NEK8 – OMIM 609799) has been found to interact with TAZ, leading to the nuclear localization of the NPHP9-TAXZ complex [45]. TAZ is normally exported to the cytoplasm through interactions with 14-3-3 proteins: NPHP9 appears to compete with 14-3-3 proteins for TAZ binding. NPHP4 inhibits LATS1 phosphorylation of TAZ, which reduces its affinity for 14-3-3 protein. NPHP4 also interacts with the ubiquitin ligase JADE-1 (another ciliary and nuclear component), an interaction that appears to stabilize JADE1 and lead to its nuclear localization where it acts to destabilize β-catenin and inhibit canonical Wnt signaling [46]. In cells, NPHP4 itself appears to be primarily perinuclear. The absence of NPHP4 activity leads to an increase in Wnt signaling and subsequent cyst formation in the kidney [46]. Studies in the zebrafish support this mechanism [47]. **NPHP7** (OMIM: 611498) is a GLI-like zinc finger transcription factor involved in the regulation of mesenchymal-epithelia cell behavior (inhibiting the

expression of genes such as Snail1 and Wnt4)[48]. Mutations in NPHP7 have been associated with NPH-related ciliopathy [49]. Morpholino-mediated down regulation of NPHP7 has been found to be influence the formation of immotile motile cilia and number of associated phenotypes, including cystic pronephros and ciliary motility in zebrafish [50, 51], presumably due to effects on gene expression.

Bardet-Biedl syndrome, a pleiotropic oligogenic ciliopathy [4] and references therein), involves (at least) nineteen different genes with potentially etiological roles. Of these, **BBS1, 2, 4, 5, 6, 7, 8 and 10** have been found to enter the nucleus where they can influence gene expression through interactions the polycomb group member protein RNF2 (Ring Finger Protein 4: OMIM:602850)[52]. Both BBS1 and BBS11 have been reported to interact with, and to alter the activity of NPHP7 [50, 51].

Centrins (Cetn) are calmodulin-like proteins associated with centrosomes (microtubule-organizing centers) and the basal body regions of cilia. Two centrin subclasses have been identified, Cetn2-like and Cetn3-like [53]. In the yeast *Saccharomyces cerevisae* there is a single centrin gene (Cdc31); the Cdc31 protein is found localized to nuclear pores (in addition to the spindle pole body)[54]. Mutations in Cdc31 lead to defects in mRNA export [55]. In vertebrate cells, Cetn2 has been found associated with nucleoporins and localized to nuclear pores; expression of the Cetn2-binding regions of NUP160 lead to a decrease in nuclear export of mRNA and protein export, without obvious effect on protein import [53]**.**

While Cetns have been reported to play a role in cell division in vertebrate cells [53], subsequent studies in chick DT40 cells [56] revealed that null mutations in all three centrin genes had no effect on cell division (although effects on cilia formation were not reported). This made it possible to examine nuclear function of centrins, two of which had been previously reported. First, Cetn2 is an integral component of the nucleotide excision repair/xeroderma pigmentosum group C (XPC-RAD23-CETN2) complex [57, 58]. Cetn2-null DT40 cells displayed *no* defects in centrosome formation or cell division but were reported to be hypersensitive to UV irradiation [53]. It should be noted that the centrin-associated protein pericentrin has also been implicated in DNA damage repair [59]. Interactions between pericentrin and microcephalin (MCPH1) have been implicated in primodial dwarfism [60], genome instability, and centrosome amplification [61].

More recently, Cetn2 has been found to be part of the nuclear pore associated TREX-2 complex (GANP, DSS1, ENY2, PCID2 & CETN2)[62]. TREX-2, in turn, has been reported to interact with the Mediator complex to link to regulate gene expression and the export of mRNAs from the nucleus [63, 64]. Our own work in *Xenopus* indicates that Cetn2 associates with sites with the promoter regions of a subset of FGF and FGF receptor genes, regulating their expression and that its morpholino-mediated down regulation leads to defects in mesoderm formation [41].

An interesting point to emerge from studies of Cetn2 is that while standard immunofluorescence microscopy reveals it to be concentrated at centrosomes and basal bodies, cell fraction studies suggest that more than 90% of centrin is soluble [53] and associated with XPC [53], a reminder that proteins can redistribute upon cell solubilization [34].

Chibby (Cby)(OMIM: 607757) is a small evolutionarily conserved protein associated with basal bodies and involved in ciliogenesis [65]. In humans there are three Cby-like proteins (Cby, Cby2, and Cby3). In vertebrate cells. but not (apparently) in *Drosophila* [65], Cby acts as a negative regulator of β-catenin-mediated Wnt signaling [66-68].[4] Cby's interaction with β-catenin involves 14-3-3 proteins and leads to β-catenin's export from the nucleus [69, 70]. STRING analysis (**FIG. 2**) indicates that Cby1 interacts with a number of 14-3-3 (YWHAX) proteins; a conclusion supported by preliminary studies in which Cby-GFP was found to co-precipitate interactions with a number of YWHAX proteins (stars in FIG. 2)(McClure-Begley et al., unpublished observations). In human induced pluripotent stem cell (HiPSC)-derived cerebral organoids, Cby is widely expressed and appears largely nuclear (McClure-Begley et al., in progress) (**FIG. 3**). Characterization of *cby*$^{-/-}$ mice (in a C57BL/6 background) showed ~75% died within

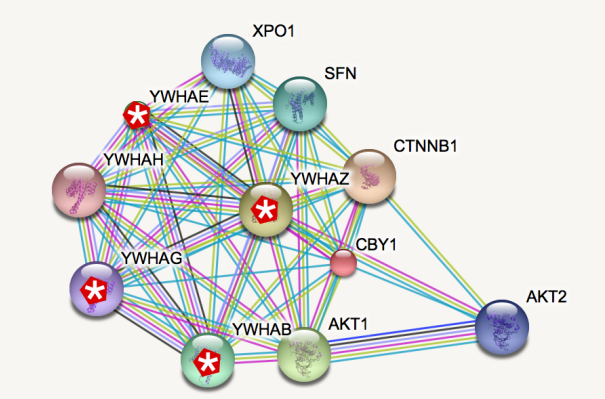

FIGURE 2: A String interaction map for Cby1 (http://string-db.org/cgi/network.pl?taskId=nnIlPEaytPOX) reveals a umber of interaction partners. 14-3-3 proteins (YHYAX) marked by * were identified as Cby-1 associated proteins in *Xenopus laevis* using an immunoisolation and mass spectrometry analysis (Shi et al., unpublished observations).

[4] Based on a simple BLAST search, there does not appear to a *C. elegans* ortholog of Chibby.

two weeks of birth and were “runted and demonstrated anemia” [71]; those animals that survived displayed a number of cilia-related defects [72]. In *Xenopus laevis*, morpholino-mediated down-regulation of Cby lead to ciliary defects and abnormal neural crest, central nervous system, and pronephros development [68]. Many, but not all, morphant phenotypes could be ameliorated by the Wnt inhibitor SFRP2, suggesting these Cby deficit phenotypes are due to increased Wnt signaling. In this light, Cby morphant effects on Hh components were not rescued by SFRP2, and so presumably represent “pure” (or at least, non-Wnt related) ciliary signaling processes [68].

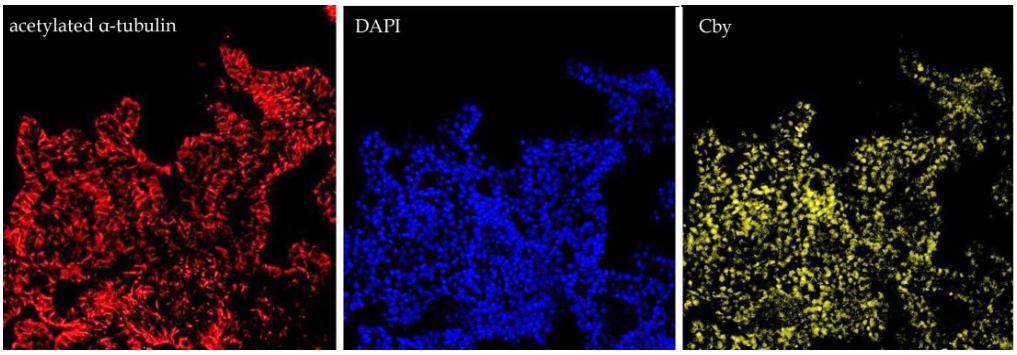

FIGURE 3: Human iPSC-derived cerebral organoids were sectioned and stained for acetylated α-tubulin (A), the DNA marker DAPI (B), and Cby (C) using a rabbit anti-Cby antibody (supplied by Feng-Qian Li (University of Stonybrook and described in reference 66.

Summary: The challenges in separating the ciliary and cytoplasmic from possible nuclear functions of CAPs are similar to those faced in the analysis of the adhesive (cytoplasmic) and gene expression (nuclear) roles of the β-catenin and γ-catenin (plakoglobin), which share many interaction partners [73]. The use of cytoplasmically anchored forms allowed us to conclude that γ-catenin’s effects on β-catenin-mediated gene expression were indirect [74]. Given the shared mechanisms acting at nuclear pores and the ciliary entry domain, a similar (or perhaps a more modern) strategy needs to be systematically employed to discern ciliary from non-ciliary, i.e. nuclear, roles of CAPs. In the case of CAPs, the problem is compounded by the fact that signaling via primary cilia is already know to engage transcriptional responses. It can be a daunting task to parse out effects of altered ciliary protein functions mediated via their impacts on ciliary assembly or signaling versus those that may be due to their impacts on genomic DNA accessibility, mitotic features, etc.

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Supplemental DATA 1: Ciliary proteins with RNS > 0.35 Xenopus data set (Wühr, Güttler et al. 2015)

ARL3 ADP-ribosylation factor-like protein 3 **RNC = 0.51 (nuc + cyto)**
interactor of RP2 and PDEdelta. Involved in trafficking of vesicles from the Golgi to the cilium, especially; farnesylated cargo in association with PDEdelta and RPGR; and myristoylated cargo in association with UNC119 and RP2 Cytosol

ARL6 ADP-ribosylation factor-like protein 6 **RNC = 0.49 (nuc + cyto)**
Mutated in Bardet-Biedl syndrome (type 3) and retinitis pigmentosa (type 55). At the ciliary gate, regulates Wnt signalling. Functions with BBSome to coat proteins for trafficking to the cilium Basal Body, Cilium, Cytosol, Transition Zone

ATXN10 Ataxin 10 **RNC = 0.48 (nuc + cyto)**
Mutated in 1 NPHP family (splice-site mutation). Forms complex with NPHP5 and 6 at the basal body

***C21orf59** (Jaffe, Grimes et al. 2016) **RNC = 0.48 (nuc + cyto)**

C8orf37 **RNC = 0.36 (nuc + cyto)**
Mutated in cone-rod dystrophy (type 16) and retinitis pigmentosa (type 64). Localizes to Basal body in cultured RPE cells and Basal body and ciliary rootlet in mouse photoreceptors Basal Body, Ciliary Root

***CETN2** Centrin-2 (Shi, Zhou et al. 2015) **RNS = 0.55 / 0.58 (nuc + cyto)**

DNAL1 Dynein light chain 1, axonemal **RNS = 0.49 (nuc + cyto)**
Mutated in primary ciliary dyskinesia (type 16) Component of outer dynein arms. Axoneme

***EFHC2** EF-hand domain-containing family member C2 **(**(Zhou, Shi et al. 2016) **RNC = 0.47 (nuc + cyto)**

HEATR2 HEAT repeat-containing protein 2 **RNC = 0.63 (nuc + cyto)**
Mutation causes PCD, presumably involved in dynein arm transport or assembly Cytosol

HSPA8 Heat shock cognate 71 kDa protein **RNC = 0.49 (nuc + cyto)**
Chaperone of the IFT together with DnajB6

HSPB11 Heat shock protein beta-11 **RNC = 0.48 (nuc + cyto)**
IFT25, part of IFT-B complex. Forms a complex with IFT27. External submission. Cilium, IFT

IFT27 Intraflagellar transport protein 27 **RNC = 0.45 (nuc + cyto)**
component of IFT complex B. Rab-like small G protein Basal Body, Cilium, IFT

MAPRE1 (EB1) MT-associated protein RP/EB family 1 **RNC = 0.38 (nuc + cyto)**
MT plus-end-tracking protein. promotes ciliogenesis. Centrosome, Cytosol, Golgi

MNS1 Meiosis-specific nuclear structural protein 1 **RNC = 0.37 (nuc + cyto)**
Mns1-/- mice have short, immotile sperm flagella, situs defects and hydrocephalus. Protein localised along flagellum. Knockdown in IMCD3 and 3T3 cells causes Hh signalling defects Axoneme

NME8 Thioredoxin domain-containing protein 3 **RNC = 0.39 (nuc + cyto)**
The sea urchin ortholog of this gene encodes a component of sperm outer dynein arms, and the protein is implicated in ciliary function. Mutations in this gene are implicated in primary ciliary dyskinesia type 6

NPHP1 Nephrocystin-1 **RNC = 0.36 (nuc + cyto)**
Known ciliopathy gene JBTS4, NPHP1, SLS1 Transition zone

NUP214 Nuclear pore complex protein Nup214 **RNC = 0.45 (nuc + cyto)**
part of ciliary pore complex Transition zone

NUP35 Nucleoporin NUP35 **RNC = 0.47 (nuc + cyto)**
nucleoporin 35kDa, part of ciliary pore complex Transition zone

NUP37 Nucleoporin NUP37 **RNC = 0.56 (nuc + cyto)**
part of ciliary pore complex Transition zone

NUP62 Nuclear pore glycoprotein p62 **RNC = 0.38 (nuc + cyto)**
part of ciliary pore complex Transition zone

NUP93 Nuclear pore complex protein Nup93 **RNC - 0.45 (nuc + cyto)**
part of ciliary pore complex Transition zone

PDE6D cGMP 3',5'-cyclic phosphodiesterase subunit delta **RNC = 0.40 (nuc + cyto)**
Part of the ARL13B, INPP5E and CEP164 network

PLK1 Serine/threonine-protein kinase PLK1 **RNC = 0.39 (nuc + cyto)**
Localizes to TZ and induces phosphorylation of NPHP1 Transition Zone

RILPL2 RILP-like protein 2 **RNC = 0.50 (nuc + cyto).**
Rab effector. Regulates cilium membrane content. Cilium, Basal Body

RSPH4A Radial spoke head protein 4 homolog A **RNC = 0.90 (most nuclear)**
Mutations cause primary ciliary dyskinesia, with typical respiratory features, but without situs abnormalities. Cilium, Axoneme

RSPH9 Radial spoke head protein 9 homolog **RNC = 0.44 (nuc + cyto)**
Similar to RSPH4A

SNX10 Sorting nexin-10 (Fragment) **RNC = 0.51 (nuc + cyto).**
regulates ciliogenesis Centrosome

SSNA1 Sjoegren syndrome nuclear autoantigen 1 **RNC + 0.50 (nuc + cyto).**
siRNA knockdown in 3 mouse cell lines perturbs receptor tranport into cilium and HH signalling.

TNPO1 Transportin-1 **RNC = 0.44 (nuc + cyto)**
Importin beta 2. Regulates entry of RP2 and kinesin motor into cilium Axoneme

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Supplemental data 2

Cilia-associated proteins found in SHSY5Y-NUCLEAR data set:

ARF4	ARL3*	ARL6*	ASAP1	ATXN10*	Cetn2*
Cetn3	CEP135	CEP250	CEP41	CEP89	CEP97
CP110	CROCC	CTNNB1**	DNAH1*	DNAH11	DNAH5
DPCD	DPYSL2	DYNLT1	EXOC3	EXOC4	EXOC5
EXOC6	FLNA	GSK3B	HEATR2*	HSPA8*	HTT
IFT27*	IFT52	IFT81	INVS	KIF3A	KIF3C
LZTFL1	MAPRE1* (EB1)	MLF1	NGFR	NME7	NPHP4
NUP214*	NUP35*	NUP37	NUP62*	NUP93*	OCRL
ODF2	ORC1	PAFAH1B1	PARD3	PCM1	PDE6D*
PHF17	PKD2	PLK1*	RAB11A	RAB23	RAB8A
RAN	RANBP1	RFX3	RP2	SEPT2	SEPT7
SGK196	SNAP25	STK38L	SYNE2	TNPO1*	TRAPPC10
TRAPPC3	TRIM32	TRIP11	TTC21B	TTC30A	TTC8
TUBA1C	TUBA4A	TUBB2A	TUBB2B	TUBB3	TUBE1
TUBGCP2	TUBGCP3	TUBGCP4	VDAC3	XPNPEP3	

* indicates also found to have a nuclear presence in the Xenopus data set

* * JUP / Plakoglobin also present