Ciliary dynein motor preassembly is regulated by Wdr92 in association with HSP90 co-chaperone, R2TP

Petra zur Lage1, Panagiota Stefanopoulou1, Katarzyna Styczynska-Soczka1, Niall Quinn1, Girish Mali2, Alex von Kriegsheim1,4, Pleasantine Mill2, and Andrew P. Jarman1

The massive dynein motor complexes that drive ciliary and flagellar motility require cytoplasmic preassembly, a process requiring dedicated dynein assembly factors (DNAAFs). How DNAAFs interact with molecular chaperones to control dynein assembly is not clear. By analogy with the well-known multifunctional HSP90-associated cochaperone, R2TP, several DNA AFs have been suggested to perform novel R2TP-like functions. However, the involvement of R2TP itself (canonical R2TP) in dynein assembly remains unclear. Here we show that in Drosophila melanogaster, the R2TP-associated factor, Wdr92, is required exclusively for axonemal dynein assembly, likely in association with canonical R2TP. Proteomic analyses suggest that in addition to being a regulator of R2TP chaperoning activity, Wdr92 works with the DNAAF Spag1 at a distinct stage in dynein preassembly. Wdr92/R2TP function is likely distinct from that of the DNAAFs proposed to form dynein-specific R2TP-like complexes. Our findings thus establish a connection between dynein assembly and a core multifunctional cochaperone.

Introduction

Many tasks within the cell are accomplished by highly organized multicomponent protein complexes, the biogenesis of which requires guided assembly by molecular chaperones (Makhlouf and Houry, 2012). Particularly striking examples are the axonemal dynein motor complexes of motile cilia. These massive multisubunit complexes are visible by electron microscopy as outer and inner dynein arms (ODA and IDA, respectively; King, 2016). The motors require a complex pathway of preassembly in the cytoplasm before their transport to the cilium (Fok et al., 1994; Fowkes and Mitchell, 1998; Mitchison et al., 2012). In recent years, a group of some 10 proteins have been identified that facilitate this assembly pathway—collectively known as axonemal dynein assembly factors (DNAAFs; Mitchison et al., 2012). Several of these proteins were discovered through identifying causative mutations in human primary ciliary dyskinesia (PCD), characterized by ciliary/flagellar immotility (Omran et al., 2008; Mitchison et al., 2012; Knowles et al., 2013; Moore et al., 2013; Tarkar et al., 2013; Zariwala et al., 2013; Diggle et al., 2014). These assembly factors are largely cytoplasmic, and when their function is impaired, ODAs and/or IDAs are missing from the ciliary axoneme. Although the specific functions of most of these assembly factors are unclear, based on protein sequence and protein interactions, several appear to recruit the ubiquitous chaperone, HSP90. HSP90 functions at late stages of protein folding (Wandinger et al., 2008; Taipale et al., 2010), and is particularly associated with the assembly of protein complexes (Boulon et al., 2010). It has a wide range of potential clients, although few have been extensively analyzed in vivo (Zhao et al., 2005; McClellan et al., 2007). Aside from PCD, protein homeostasis is increasingly associated with diseases from neurodegeneration to cancer, and is a promising target for therapeutic intervention (Lindquist and Kelly, 2011).

In Drosophila melanogaster, the entire repertoire of known DNAAFs is conserved (unpublished data), but the fly has only two cell types bearing cilia with motile characteristics: the sperm flagellum and the ciliated sensory dendrite of auditory/proprceptive chordotonal (Ch) sensory neurons (Cachero et al., 2011), in which ciliary motility seems to be critical in mechanosensory transduction (Karak et al., 2015). Therefore, mutations of DNAAF genes specifically result in auditory/proprceptive deficiency and male infertility (Kavlie et al., 2010; Moore et al., 2013; Diggle et al., 2014). The restricted expression and function of motile cilium genes in Drosophila accelerates discovery and characterization of new motility-related genes: a screen for such genes led to the discovery of PCD-causative genes, ZMYND10 and DNA AF5/HEATR2 (Moore et al., 2013; Diggle et al., 2014). In the same screen, we identified CG14353, which encodes a homologue of the human WD40-repeat protein, WDR92 (also known as MONAD; CG14353 is hereafter referred to as Wdr92). WDR92 is poorly characterized functionally, although overexpression studies in cell
culture suggested a link to apoptosis (Saeki et al., 2006). WDR92 is known best from several proteomic studies through its binding to the RPAP3 subunit of the R2TP cochaperone complex (Sardiu et al., 2008; Glatter et al., 2011; Cloutier et al., 2017), which is potentially of great significance for dynein preassembly. R2TP brings clients to the chaperones HSP70/HSP90 in the assembly and/or stabilization of a variety of protein complexes from yeast to human (Boulon et al., 2010, 2012), including small nuclear RNA-protein complexes (snoRNPs), phosphatidylinositol-3 kinase-related protein kinase (PIKK)–containing complexes (e.g., mTOR), and RNA polymerases (Kakihara and Houry, 2012; von Morgen et al., 2015). Although disruption of the R2TP-associated helicases Reptin (Ruvbl2) or Pontin (Ruvbl1) causes phenotypes associated with impaired cilia motility in zebrafish (Zhao et al., 2013), this has been interpreted as reflecting their participation with certain DNAAFs in cilium-specific alternative R2TP-like complexes (Tarkar et al., 2013; Pal et al., 2014; Vaughan, 2014; Li et al., 2017; Olcese et al., 2017; Paff et al., 2017). In contrast, “canonical” R2TP itself has not been directly linked to dynein assembly. In addition, in proteomic analyses, WDR92 is consistently found associated with the prefoldin-like complex (Sardiu et al., 2008), which by analogy to the canonical prefoldin complex is proposed to have chaperone activity (Millán-Zambrano and Chávez, 2014). However, the functional significance of this association is unknown.

Comparative genomics demonstrate that WDR92 genes are specifically associated with organisms that bear motile cilia (Baron et al., 2007), and indeed, recent research supports a role in planarian ciliogenesis, although its function was not defined (Patel-King and King, 2016). To test the hypothesis that Wdr92 (and R2TP) functions in dynein preassembly, we explored its function in Drosophila. We show that Drosophila Wdr92 is a cytoplasmic protein exclusively expressed in motile ciliated cells and is required exclusively for ciliary/flagellar motility. The major effect of its mutation is loss of dynein arms from the axonemes of sensory neuron cilia and sperm flagella. We show that Wdr92 associates with the DNA AF Spagl, confirm that Drosophila Wdr92 also interacts with R2TP, and show that R2TP depletion also impairs dynein arm formation. We show that Wdr92 protein is associated with both dynein heavy chains (HCs) and intermediate chains (ICs), and propose that it acts as a specificity factor to bring partially assembled dynein clients to R2TP/HSP90 at a late stage of cytoplasmic assembly. Thus, Drosophila Wdr92 is a new DNAAF that strongly reinforces the critical role of HSP90 and cochaperones in dynein assembly.

Results
Drosophila Wdr92 is expressed in developing Ch neurons and sperm
FlyAtlas adult expression data show that Wdr92 is highly and specifically expressed in testes (Robinson et al., 2013). Testis expression is confirmed by RNA in situ hybridization, which shows expression in round spermatocytes but not mature sperm (Fig. 1 A). In embryos, Wdr92 mRNA is present in differentiating Ch neurons and their precursors (Fig. 1, B and C). Moreover, this expression is strongly reduced in embryos bearing a mutation in Rd3F, which encodes a FOXJ1-orthologous transcription factor that regulates cilia motility genes in cooperation with the ciliogenic transcription factor, Rfx (Fig. 1 D; Cachero et al., 2011; Newton et al., 2012). Consistent with being an Rd3F/Rfx target gene, the Wdr92 5′UTR has a conserved pair of Rfx and Rd3F binding motifs common to other target genes (Fig. 1 E).

Expression exclusive to Ch neurons was confirmed in embryos expressing a Wdr92-mVenus fusion protein reporter (present as a transgene driven by the Wdr92 promoter; Fig. 1, F–H). In the pupal antenna, Wdr92-mVenus expression was detected in the differentiating Ch neurons that comprise Johnston’s organ (required for hearing and proprioception), but not in other ciliated (but nonmotile) sensory neurons (Fig. 1, I–K). Fusion protein expression was also detected in the tests within developing spermatocytes but not in mature sperm (Fig. 1 L). In all cases, expression of the fusion protein was not observed in the cilium/flagellum but was confined to the cytoplasm. Thus, Wdr92 is a cytoplasmic protein expressed during the development of the only two cell types that bear cilia/flagella with motile features.

Wdr92 is required for Ch neuron function and sperm motility
In initial experiments, we depleted Wdr92 expression using flies with two independent Gal4-inducible RNAi constructs. When depleted in developing Ch neurons (using a UAS-Dcr2; scaGal4 driver line), resulting adult flies showed defective behavior in a climbing assay (Fig. 2 A). Combined with Ch-neuron–specific expression, this indicates defective proprioception resulting from impaired Ch neuron function in adult antennae and legs. Depletion in the male germline (BamGal4) resulted in complete infertility (n = 10 males; Fig. 2 B).

These phenotypes were confirmed in a null allele of Wdr92 generated by CRISPR/Cas9 gene replacement of the entire ORF with a mini-white gene (Wdr92−/−, henceforth referred to as Wdr92+/−; Fig. 2 C). Wdr92−/− flies were viable with no visible defects, indicating that Wdr92 is not required for general cell viability. However, the flies exhibited poor proprioception in a climbing assay, and this was rescued by the Wdr92-mVenus fusion gene (Fig. 2 D). Moreover, the auditory function of larval body wall Ch neurons was defective in a behavioral assay for larval hearing (Fig. 2 E). Wdr92−/− males were completely infertile. Testes dissected from Wdr92−/− males had sperm bundles clearly visible, but no motile sperm were observed in the seminal vesicles (SVs), and none were released after crushing of the testes, in contrast to controls (n = 10 males; Fig. 2, F and G).

Wdr92 is required for the presence of axonemal dynein arms
Immunofluorescence analysis of Wdr92−/− embryos and pupal antennae revealed grossly normal Ch neuron structures, and the presence of the sensory cilium appeared unaffected (Fig. 3, A and B). Transmission electron microscopy (TEM) confirmed a grossly normal ultrastructure of the Ch neuron sensory cilium, with normal basal body, transition zone, ciliary dilation, and ciliary rootlet (unpublished data). However, transverse sections of the proximal cilium in both deletion and depletion mutants showed a complete absence of the ODA/IDA normally observed in this region (Wdr92−/−: 16/16 cilia showed complete loss of arms; Wdr92−/− heterozygote control: 14/14 cilia showed normal arms) (Fig. 3, E–G). To confirm this phenotype, we examined the
localization of a dynein subunit, Dnali1/Ca6971, using a Dnali1-mVenus line (Diggle et al., 2014). When IDA assembly occurs normally, Dnali1 is located in the proximal motile zone of Ch neuron cilia (pz in Fig. 3 C), but it was completely absent from Wdr92−/− cilia (Fig. 3 D). In contrast, the mechanosensory ion channel NompC was correctly localized in the distal (nonmotile)
zone of the cilium (Fig. 3, C and D). Therefore, by ultrastructure and immunofluorescence analysis, Wdr92 mutation specifically affects dynein motors. This contrasts with planarian Wdr92, the depletion of which resulted in diverse defects in ciliogenesis (Patel-King and King, 2016).

TEM analysis of sperm bundles in the adult testis also showed a lack of dynein arms in both Wdr92 deletion and depletion mutant and testes (Fig. 3, H–J). Apart from this, sperm bundles seemed largely unaffected (with ∼64 spermatozoids per bundle), although sperm individualization may be impaired as was reported for a dynein chain mutation (Fatima, 2011). In addition to dynein arm loss, axonemal A microtubules were sometimes occluded, and individual microtubule doublet complexes sometimes separated from the rest of the axoneme (Fig. 3 J). These features had been previously noted for Drosophila homologues of several DNAFs, notably tilB/LRRC6 (Kavlie et al., 2010), Zmynd10 (Moore et al., 2013), Heatr2 (Diggle et al., 2014), and Dnaaf3/CG17669 (unpublished data). Interestingly, occluded A tubules were also observed for planarian Wdr92 knockdown (Patel-King and King, 2016). In wild-type testis, Dnali1-mVenus strongly localizes to the maturing flagellar axonemes in the sperm bundles (Fig. 3K). This is disrupted in Wdr92−/− testes—Dnali1-mVenus protein is not localized along the flagella, but is present in the vicinity of the flagella in aggregates (Fig. 3L). This difference in Dnali1-mVenus localization between sperm and Ch neurons might reflect...
differences in dynein transport during ciliogenesis: in Ch neuron ciliogenesis, transport is likely dependent on intraflagellar transport, but sperm flagellum synthesis proceeds within the cytoplasm in a non-intraflagellar transport–dependent mechanism (Han et al., 2003).

**Dynein HC and IC abundances are reduced in Wdr92 mutant round spermatocytes**

To pinpoint when in cytoplasmic dynein assembly WDR92 function is required, we performed unbiased label-free mass spectrometry (MS) quantification of proteins in control versus Wdr92−/− mutants. We used extracts from 48-h pupal testes, a stage before sperm flagellum formation (Gärtner et al., 2014), to detect changes primarily reflecting Wdr92’s cytoplasmic role in dynein assembly/transport rather than secondary proteostatic effects of failure to populate flagella with motors. Interrogation of the MS data with a candidate list of 88 motility-associated proteins revealed that 40 could be detected in spermatocytes, and 20 of these appeared altered in abundance in mutant spermatocytes (P < 0.05; Fig. 4 A and Tables S1 and S2). Strikingly, these include a reduction in abundance of six dynein HCs representing all forms of ODA/IDA (ODA β and γ chains, IDA heterodimeric and...
monomeric forms) and several IDA ICs. Reduction is consistent with destabilization caused by failure in assembly of HC/IC complexes (Mitchison et al., 2012). The phenotype is notably different from mutants of several known DNAAFs, including DNAAF2, DNAAF3, and ZMYND10, in which HCs are reduced but ICs accumulate (Omran et al., 2008; Mitchison et al., 2012; Diggle et al., 2014).

Interestingly, several DNAAF orthologues were increased in the Wdr92 mutant. Dnaaf2/Ktu (Nop17l in Drosophila) and Dyx1c1/Dnaaf4 (CG14921), which are proposed to form an R2TP-like cochaperone complex for an early HC assembly step, are both strongly increased. C21orf59/Kurly (CG18675) and Heatr2 (CG31320) are also increased. Surprisingly, there are reductions in homologues...
of dynein motor docking proteins: Ccdc63 (CG17083) and Armc4 (Gudu; ODA docking complex proteins), and two Tektns, which are implicated in IDA docking in Chlamydomonas reinhardtii and mouse (Tanaka et al., 2004; Yanagisawa and Kamiya, 2004).

Wdr92 protein interacts with R2TP, prefoldin-like complex, and dynein chains

To obtain insight into the link to dynein motors, we determined the Wdr92 interactome in fly testes. Using GFP-trap affinity purification (AP), we immunoprecipitated Wdr92 from testes of Wdr92-mVenus adult males. As a negative control to exclude mVenus-interacting proteins, we performed AP-MS on extracts from testes overexpressing a ubiquitous, cytoplasmic Gap43-mVenus fusion protein. Proteins showing significant association with Wdr92 included several categories of interest for motor assembly, including known DNAAs and PCD-causative genes, and dynein chains (Fig. 4 B and Table 1). First, AP-MS confirmed Wdr92 association with predicted R2TP and prefoldin-like complexes, reflecting previous proteomic analyses of human WDR92 interactors in cultured cells (Sardiu et al., 2008; Choi et al., 2011; Cloutier et al., 2017; Fig. 8).

Critically, AP-MS provides the first evidence that Wdr92 associates with ciliary motility proteins. This includes dynein chains, which may be clients for Wdr92/R2TP cochaperone activity, and DNAAs homologues, which may be functional partners. Dynein chains include HC and ICs of all forms of ODA and IDA. A single light chain is associated with Wdr92: the homologue of DNA4. This is a component of the ODA, but it is also known that mutation of the homologous subunit in Chlamydomonas (LC10) causes defects in ODA assembly (Tanner et al., 2008). Surprisingly, two radial spoke proteins are present among the interactors (RSPH4A/6A and RSPHI). The protein interaction data provide evidence supporting a role for Wdr92 in bringing dynein client proteins to the R2TP cochaperone.

R2TP complex is required for dynein arm assembly; prefoldin-like complex is not

R2TP comprises two parts: a heterodimer of Rpap3 and Pih1d1 (Spaghetti and CG5792 in Drosophila (Benbahouche et al., 2014), and a heteromultimer of helicases RUVBL1/Pontin and RUVBL2/Reptin, although these latter additionally play many non-R2TP roles (Sardiu et al., 2008; Choi et al., 2011; Kakhkara and Houry, 2012; Nano and Houry, 2013; Cloutier et al., 2017; Fig. 8). In coimmunoprecipitation (coIP) experiments of Drosophila S2 cells transfected with constructs to overexpress tagged proteins, we found that Drosophila Wdr92 physically interacts with Rpap3/Pih1d1, confirming the AP-MS results above and consistent with the known human interactions (Sardiu et al., 2008; Choi et al., 2011; Cloutier et al., 2017; Fig. 4 C). It is likely that Wdr92 binds Rpap3/Spaghetti directly via the latter’s C-terminal RPAP_3C domain; however, in S2 cells, Wdr92 associated with Pih1d1 as well as Rpap3, probably through bridging via endogenous Rpap3 (Fig. 5I). Although we did not detect Reptin or Pontin as Wdr92 interactors by AP-MS, we could confirm interaction with Pontin/Reptin in S2 cells (Fig. 4 C). These interactions may similarly be facilitated by endogenous Rpap3 protein.

Consistent with the complex’s multiple roles, Drosophila homologues of R2TP subunits are not strongly enriched in Ch neurons, and mutations are lethal (Table S3). To investigate a possible role for R2TP in dynein motor assembly and to overcome cell-vital functions, we performed tissue-specific RNAi depletion in spermatocytes for the R2TP-unique subunits, spaghetti/Rpap3 and CG5792/Pih1d1. In each case, we observed complete male infertility (n = 10 males) with testes that appeared to contain largely normal sperm bundles but lacked motile sperm (n = 7 for CG5792, n = 11 for spaghetti; Fig. 5, A–C). TEM confirmed relatively normal sperm bundles (Fig. 5 J) and flagella (Fig. 5, D–F) but with strong loss of dynein arms as well as the occurrence of A tubule occlusions and axonemal fragmentation (Fig. 5, D–F).

Depletion of either gene in developing sensory neurons also resulted in normal ciliature appearance but with loss of dynein arms (Fig. 5, G–I), although this was incomplete for CG5792 (spaghetti: 9/12 cilia showed loss of arms; CG5792: 7/20 cilia showed partial loss of arms). When propropoception was assayed in flies raised at 28.5°C (enhancing the efficiency of RNA depletion), a stronger behavioral defect was observed for both subunits (Fig. 5 K), suggesting that RNAi is inefficient for this line. These phenotypes suggest that a major role of R2TP in motile ciliated cells is to promote dynein arm assembly.

Despite their widespread functions, both reptin and pontin are enriched in Ch neurons, suggesting a stronger requirement than in other cells (Fig. 6, A and B), and the enriched expression of reptin in Ch neurons appears to be partially dependent on the motile cilia transcriptional regulator, Fd3F (Fig. 6 C). RNAi depletion of either reptin or pontin in Drosophila testes indeed resulted in infertility (Fig. 6 G) and specific absence of motile sperm (n = 10 flies) (Fig. 6, D and E), suggesting that their major role in sperm differentiation is concerned with dynein motors. We were unable to assess the function of these genes in Ch neurons, because embryonic depletion of either gene proved to be lethal.

The AP-MS data show that Wdr92 strongly associates with the five subunits of the prefoldin-like complex (Fig. 4 B), in agreement with interactions previously noted for Drosophila and human WDR92 (Sardiu et al., 2008; Glatter et al., 2011). The prefoldin-like complex shares two subunits (RSPH4A/6A and RSPHI) with the canonical prefoldin complex, which is best known for assisting in the folding of actin and α- and β-tubulin through interaction with the Chaperonin-containing TCP-1 complex (Millán-Zambrano and Chávez, 2014). Three subunits are unique to the prefoldin-like complex (UR11, PDRG1, UX1T), which we investigated here. Surprisingly, RNAi depletion of each of these subunits resulted in neither propropoceptive defects (when depleted in sensory neuron precursors) nor male infertility nor sperm immotility (when depleted in spermatocytes; Uri, 4 independent RNAi lines; Uxt, 3 lines; Pdrg1/CG15863, 1 line; for sperm motility, n = 10 males for each line). This suggests that despite its proteomic link with Wdr92/R2TP, the prefoldin-like complex is not involved in dynein arm assembly. Moreover, unlike Wdr92, there is no indication that expression of Drosophila prefoldin-like genes is enriched in ciliated cells, either in the Ch neuron transcriptome or in testis (Table S3).
Wdr92 interacts with CG18472, an orthologue of DNAAF, Spag1

By AP-MS, the orthologues of two DNAAFs associate with Wdr92. This includes CG18472, the closest Drosophila homologue of human SPAG1 (Knowles et al., 2013; 49% similarity). Like RPAP3, human SPAG1 has an N-terminal TPR that may associate with HSP90 and a C-terminal potential WDR92-binding RPAP3_C domain. Indeed, recent proteomic analysis supports an association between human WDR92 and SPAG1 (Cloutier et al., 2017). However, the C-terminal domain is not conserved in Drosophila CG18472 (Fig. 7A); nor in other insect homologues. Despite this difference, we obtained several lines of evidence that suggest the CG18472 protein conserves the dynein assembly function of human SPAG1. First, CG18472/Spag1 mRNA is highly expressed in the differentiating Ch neuron transcriptome (17.3-fold enriched; Cachero et al., 2011) and in testis. Flies in which CG18472/Spag1 was depleted in Ch neurons had defective larval hearing (Fig. 7B) and adult proprioception (Fig. 7C), whereas depletion in testes caused complete male sterility (Fig. 7D) with mature but immobile sperm (n = 8 flies; Fig. 7, E and F). In addition, by TEM, Ch neuron cilia, and sperm flagella lacked ODA/IDA (22/25 cilia showed complete loss of arms; Fig. 7, G and H). We conclude that CG18472/Spag1 likely conserves the dynein assembly function of human SPAG1.

To explore the Wdr92-Spag1 association, we conducted AP-MS on Spag1 from testes of adult males with a Spag1-mVenus fusion gene. Strikingly, Wdr92 showed significant association (ratio vs. control: 11.1; P = 0.003711). The only dynein-related protein showing significant association with Spag1 was Dhc98D (Dnah10), an inner arm dynein HC that was also associated with Wdr92 (ratio vs. control: 2.4; P = 0.021265) and strongly reduced in Wdr92

Table 1. Protein interactors of Wdr92

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<th>Drosophila gene</th>
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LC, light chain; PFDL, prefoldin-like complex.

aFold difference Wdr92 AP versus control AP (p-value).
bHomologous interaction reported for human WDR92 in cultured nonmotile ciliated cells (Choi et al., 2011).
mutant spermatocytes (Fig. 4 A). Interestingly, in S2 cell coP experiments, Spag1 association with Wdr92 was facilitated by the presence of overexpressed Pontin and Reptin (Fig. 7 I). We suggest that Wdr92-Spag1 form part of a conserved cochaperone complex. It is notable that Spag1 protein abundance is decreased in Wdr92−/− testes (Fig. 4 A), consistent with codependence of the two proteins in a complex.

Discussion

Although WDR92's proteomic association with the HSP90 cochaperone, R2TP, has been known for some time, very little was known of the function of WDR92 or the significance of this association in vivo (Sardiu et al., 2008; Boulon et al., 2010; Choi et al., 2011; Glatter et al., 2011). We found that Drosophila Wdr92 protein is specifically expressed in motile ciliated cells before their terminal...
differentiation and is confined to the cytoplasm, where dynein preassembly takes place. Null mutants are viable, with specific loss of ODA and IDA from otherwise normal cilia/flagella. In these features, Wdr92 resembles fly homologues of known DNA AFs (Kavlie et al., 2010; Moore et al., 2013; Diggle et al., 2014), suggesting that Wdr92 in Drosophila is a dedicated DNA AF (Fig. 8).

R2TP is the most complex HSP90 cochaperone, with its PIH1D1 subunit thought to impart specificity for a variety of clients including TOR, snoRNP, and RNA polymerase II (Eickhoff and Costa, 2017). We provide the first direct evidence that dynein chains are also R2TP clients. Previous studies of R2TP could not reveal interactions with dynein chain clients or DNA AFs because proteomic analyses were performed on cell lines lacking motile cilia. Mutations of spaghetti (which encodes the RPAP3 subunit of Drosophila R2TP) are lethal: spaghetti null mutant embryos survive owing to maternal contribution, but the larvae then die with atrophy of most organs, which can be explained by its multifunctional cochaperone role (Benbahouche et al., 2014). In contrast, Wdr92 function appears confined to dynein assembly, suggesting that it is an adaptor specifically required to regulate the dynein assembly function of R2TP. We propose that the DNA AF, SPAG1, functions in close association with WDR92, although it remains to be determined whether WDR92-SPAG1 work together with R2TP in a single complex or WDR92-SPAG1 form an independent cochaperone complex.

Based on their possession of TPR and PIH domains, several DNA AFs have been speculated to form cochaperone complexes akin to R2TP (known as R2TP-like complexes), including DNA AF4-DNA AF2 and DNA AF4-PIH1D3 (Tarkar et al., 2013; Pal et al., 2014; Vaughan, 2014; Olcese et al., 2017; Paff et al., 2017). Moreover, studies of RU VBL1/2 requirement for dynein assembly are interpreted as evidence for their involvement in these hypothetical cilium-dedicated R2TP-like complexes (Zhao et al., 2013; Li et al., 2017). It is notable that we found no evidence of physical interaction between Wdr92 and these DNA AFs by AP-MS (nor by direct coIP experiments in S2 cells; unpublished data), suggesting that Wdr92/R2TP is a distinct entity in the dynein assembly pathway. Although evidence for R2TP-like complexes currently remains inconclusive, our study establishes that canonical R2TP is required for dynein assembly. It is possible, therefore, that previous studies of RU VBL1/2 requirement in dynein assembly indicate their involvement with canonical R2TP. Interestingly, DNA AF4 and DNA AF2 proteins were both increased in abundance in Wdr92 mutant spermatocytes, perhaps in attempted compensation for loss of Wdr92-mediated cochaperone activity.

In mutant analyses of several DNA AFs (notably DNA AF2 and DNA AF3), reductions in dynein HC abundances have been interpreted as representing instability and clearance of partly assembled motor complexes caused by incorrect HC chaperoning, whereas increase in IC abundances represents accumulation of an IC-based subcomplex before its assembly with HC s (Omran et al., 2008; Mitchison et al., 2012; Moore et al., 2013). Wdr92 mutants are unique in exhibiting reductions in HC s but no accumulation of IC s (indeed, many IC s are reduced), and Wdr92...
physically associates with both HCs and ICs. This suggests that it defines a distinct step in dynein assembly, perhaps at a late stage in the process. Several surprising associations with proteins involved in aspects of axonemal motor docking may suggest also a role in late stage complex handover for trafficking to the cilium. This includes physical interaction with radial spoke proteins that interact with docked IDAs, as well as reduced abundance in the Wdr92 mutant of outer arm docking complex subunits (ODA-DC) and tektins, which are implicated in inner arm docking in Chlamydomonas and mouse (Tanaka et al., 2004;...
Yanagisawa and Kamiya, 2004). Because tektins are also thought to line the lumen of the microtubule doublets, it is possible that disruption in tektin assembly/trafficking underlies the occluded A tubule phenotype seen in mutants of Wdr92, R2TP, and other DNAAF homologues in Drosophila.

Proteomic analyses (including the present study) consistently demonstrate coassociation of WDR92 with the prefoldin-like proteins, and on this basis, Wdr92 is often considered to be part of a prefoldin-like complex (Millán-Zambrano and Chávez, 2014). It is therefore curious that we found no functional evidence for involvement of the prefoldin-like complex in dynein preassembly, in contrast to R2TP. In fact, there is no functional evidence that human WDR92 is required for prefoldin-like functions. None of the Drosophila subunits of the prefoldin-like complex are enriched in motile ciliated cells, and mutations in their genes are lethal, suggesting a role in multiple pathways (uri, uxt/l(2)35Cc, and Pdrg1/l(3)01239 are all lethal mutations). Perhaps consistent with a lack of involvement in dynein assembly, previously it was shown that Drosophila uri mutants with a partially rescued soma function exhibit sperm defects but still produce motile sperm (Kirchner et al., 2008). In addition, only Wdr92 is phylogenetically associated with organisms with motile cilia (Baron et al., 2007). For these reasons, we suggest that Wdr92, at least in Drosophila, is not a core component of a prefoldin-like complex. Moreover, the existence of a prefoldin-like complex and its function as a chaperone are unclear. The proposal of a prefoldin-like complex as a chaperone derives largely by analogy with the canonical prefoldin chaperone complex (Millán-Zambrano and Chávez, 2014), with which it shares two subunits (FFDN2 and PFDN6). Apart from its proteomic association with protein folding/stability processes (e.g., RNA polymerase assembly, PIKK stabilization), there is so far little direct functional evidence for involvement (although the URI homologue, bud27, participates in RNA polymerase II assembly in yeast; Mirón-García et al., 2013). In contrast, functional evidence suggests several prefoldin-like subunits act independently in several processes, such as transcriptional elongation (Millán-Zambrano and Chávez, 2014; Mirón-García et al., 2014).

In human and mouse, WDR92 appears to be widely expressed and not restricted to tissues with motile cilia. Indeed, in mammals, the proteomic association of WDR92 with R2TP/prefoldin-like extends to cells without motile cilia (Mita et al., 2013). It is possible that mammalian WDR92 has nonmotile cilia roles, with or without a prefoldin-like complex. Nevertheless, WDR92 (and RPAP3) appear with highest abundance in mouse testis just before flagellogenesis (http://fantom.gsc.riken.jp/5/sstar/EntrezGene:103784). Combined with the comparative genomic link between Wdr92 and ciliary motility, it is plausible to propose that dynein assembly is a conserved major function of WDR92 in other organisms including humans, even if not the sole function.

Materials and methods

Fly stocks

Flies, unless stated otherwise, were maintained on standard cornmeal agar media at 25°C. The stocks UAS-Dcr-2; scaGal4 and the Cas9 injection line vasa::Cas9 (Bla51323) were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN). RNAi lines (Table S4) were obtained either from Bloomington (TRIPI lines) or from the Vienna Drosophila Resource Centre. BamGal4-VP16 stock was a gift from H. White-Cooper (Cardiff University, Cardiff, Wales, UK). Control flies were the appropriate RNAi line parent stock (Table S4) or Oregon-R. Other stocks used were EdF1 (Newton et al., 2012) and Dnalit-mVenus (CG6971-mVenus; Diggle et al., 2014).

Behavioral assays

The adult climbing assay and larval hearing assay were performed as previously described (Diggle et al., 2014). A 20-cm tube was divided into four sections and flies scored according to the section climbed in 15 s. Adults were tested in replicate batches of 10–15 with the mean representing the climbing index. n = 5 batches unless otherwise stated. Larvae were tested in replicate batches of five for contraction response before and during a tone of 1000 Hz, and the number of responding larvae were
aggregated as the response score for that replicate. \( n = 9 \) replicates unless otherwise stated. Data are plotted as box plots with whiskers representing minimum and maximum values.

**Male fertility assay**

RNAi flies were crossed to BamGal4-VP16. From the progeny, 2- to 5-d-old males \( (n = 10) \) were crossed to Oregon-R females and allowed to deposit eggs for 2 d. Flies were then transferred to new vials to lay eggs for 2 d. Progeny from the latter were counted. Data are plotted as bars with mean and SD.

**RNA in situ hybridization**

DIG-labeled UTP (DIG RNA Labeling Mix; 11277073910; Roche) antisense RNA probes were generated using T7 RNA polymerase (10881767001; Roche) from a PCR product (400–500 bp) containing the T7 RNA polymerase promoter at its 3’ end. Embryos or testes [Morris et al., 2009] were fixed in 4% formaldehyde and prehybridized (50% deionized formamide [vol/vol], 5× SSC, 10 mg/ml tRNA, 50 mg/ml heparin, and 0.1% Triton X-100, pH 6.5). Hybridization was performed at 70°C overnight. Tissues were washed in PBS with 0.1% Igepal CA-630 (Sigma-Aldrich). After incubation with anti–digoxigenin-AP antibody (1:2,000; 1093274910; Roche), the staining pattern was visualized by incubating in reaction buffer (100 mM Tris, pH 9.5, and 100 mM NaCl with the NBT/BCIP color reagent; 11681451001; Roche). After washing, tissues were mounted in 80% glycerol.

**Immunofluorescence**

For tissue stainings, tissues were fixed in 4% formaldehyde for 10–20 min [Newton et al., 2012], washed in phosphate buffered saline with 0.3% Triton X-100 (PBT), then blocked in PBT with 2% bovine serum albumen for 2 h. Tissues were then incubated with primary antibody in PBT overnight, washed in PBT, and incubated with secondary antibody for 2 h. After washing, tissues were mounted in VectorShield (Vector Laboratories). Antibodies used were rabbit anti-GFP (1:500; A-11122; Thermo Fisher Scientific), mouse anti-Futsch/mAb-22C10 (1:200; Developmental Hybridoma Bank Iowa), rabbit anti–HRP (1:500; Jackson Laboratories), and mouse anti-NompC (1:200; a gift from X. Liang, Yale University, New Haven, CT; Liang et al., 2011). Secondary antibodies (Thermo Fisher Scientific), Alexa Fluor 488 goat anti–rabbit (A-11008), Alexa Fluor goat anti–mouse 488 (A-11001), Alexa Fluor 568 goat anti–rabbit (A-11036), and Alexa Fluor 568 goat anti–Mouse (A-11019) were used at a concentration of 1:500. TO-PRO3 (Thermo Fisher Scientific) was used at 1:1,000.

**Fluorescence and brightfield microscopy**

Fluorescence images were acquired using a Zeiss LSM510 confocal system with AxioSkop2 or Axiointer microscope, using Zeiss LSM510 software with the following objectives: Plan Neofluor 10×/0.3, Plan Neofluor 20×/0.5, and Plan Apochromat 63×/1.4 Oil. Brightfield images were acquired using a Olympus Provis AX-70 microscope with an Olympus DP50 camera, with the following objectives: UPplanApo 10×/0.4 and UPplanApo 20×/0.7. In all cases, images were processed for gamma adjustment using FIJI software.

**TEM**

Whole adult heads were removed and rinsed in 0.5% Triton X-100. The proboscis was removed to facilitate infiltration of the fixative, and the heads were then fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C. Heads were then washed in 0.1 M phosphate buffer, pH 7.4, postfixed with OsO₄, dehydrated in an ethanol series, and embedded in Polybed812. Ultrathin (75 nm) sections of the antennae were then stained with aqueous uranyl-acetate and lead citrate and examined with a Hitachi 7000 electron microscope.

**mVenus fusion gene construction**

A Wdr92-mVenus fusion gene was designed as follows: the Wdr92 gene was PCR-amplified from genomic DNA using primers designed to include the upstream region containing predicted Rfx and Fd3F binding sites to allow expression from its own promoter. The PCR fragment was cloned into pDONR221 via a BP Gateway reaction (Life Technologies). A Gateway LR clonase reaction then transferred this insert into the pBID-UASC-GV destination vector [Wang et al., 2012] to generate vector pBID-UASC-CG14353::mVenus. Transformant fly lines were generated by microinjection into syncytial blastoderm embryos of the attP40 landing site line. A similar strategy was used for Spag1-mVenus generation. Primers are listed in Table S4.

**AP and identification of proteins from testes**

Three replicates of 150 pairs of testes were dissected from Wdr92-mVenus or Spag1-mVenus adults, the wild-type control line w1118, and the mVenus control line w1118c. adult males were crossed to Oregon-R females and allowed to deposit eggs for 2 d. Progeny from the latter were counted. Data are plotted as box plots with mean and SD.

**Protein expression analysis of testes by MS**

For protein abundance experiments, 48-h pupal testes were dissected from 15 pupae per replicate for Wdr92X2 mutant or control line w1118, and the mVenus control line w1118c. 11681451001; Roche). After incubation with anti–digoxigenin-AP antibody (1:2,000; A-11122; Thermo Fisher Scientific). Raw data were searched with MaxQuant against the Drosophila reference proteome with M(ox) and N-terminal acetylation as variable modifications. Protein quantification was done by MaxLFQ (Wisniewski and Mann, 2012). For imputation and statistical analysis, the Perseus software suite was used.
were then incubated for 30 min at 50°C. Reduced cysteine residues were alkylated by adding iodoacetamide solution to a final concentration of 50 mM and incubated 30 min at room temperature, in the dark. Proteins were digested with adding 0.1 μg Trypsin (Promega) per sample for 16 h at 37°C. Trypsin activity was inhibited by acidification of samples to a concentration of 1% trifluoroacetic acid. Digests were clarified by centrifugation (20,000 g, 5 min), samples were desalted on a C18 Stage tip, and eluates were analyzed by HPLC coupled to a Q-Exactive Plus mass spectrometer as described above but with an extended gradient of 120 min. Peptides and proteins were identified and quantified with the MaxQuant software package (1.5.7.4), and label-free quantitation intensities were performed by MaxLFQ (Wiśniewski and Mann, 2012). The search included variable modifications for oxidation of methionine, protein N-terminal acetylation, and carbamidomethylation as fixed modification. The false discovery rate, determined by searching a reverse database, was set at 0.01 for both peptides and proteins. All bioinformatic analyses were performed with the Perseus software. Intensity values were log-normalized, and $t$-values were imputed by a normal distribution $t = \frac{x - \mu}{\sigma}$ down of the mean and with a width of 0.2 $\pi$. The MS proteomics data have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD006935.

**Transfection and coIP of S2 cells**
cDNAs were synthesized from antennal or testis mRNA (a gift from F. Newton, University of Edinburgh, Edinburgh, Scotland, UK) and cloned into the C-terminal site of plasmids pAWH (3x HA epitopes) and pAWF (3x FLAG epitopes) of the *Drosophila* Gateway Vector collection (Carnegie Institution for Science). Primers for synthesis are in Table S4. Transfection into S2 cells was performed using X-TREME GENE HP DNA transfection reagent (Roche). The cells were harvested after 48–72 h, and coIP was performed according to the FLAG Immunoprecipitation kit (Sigma-Aldrich). After Western blotting, the polyvinylidene fluoride membrane was incubated with mouse anti-FLAG M2 antibodies (1:1,000; F1804; Sigma-Aldrich) and rabbit anti-HEEL (1:4,000; ab9110; Abcam) antibodies. Secondary antibodies were supplied by Li-COR (IR Dye 680RD and IR Dye 800CW), and protein detection was performed on a Li-COR Odyssey scanner using ImageStudio v5.2 software.

**Statistical analysis**
Data were plotted and analyzed using Prism 7 (Graphpad Software, Inc.). For climbing and hearing assay data, significance was determined by the Kruskal-Wallis test, with Dunn’s test for multiple comparisons. For male fertility data, significance was determined by ordinary one-way ANOVA, with Dunnett’s test for multiple comparisons; data distribution was assumed to be normal, but this was not formally tested. Significance on plots is signified by asterisks: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

**CG14353 CRISPR mutant generation**
A CG14353 CRISPR/Cas9 mutant (Wdr92<sup>2321</sup>) was constructed by mini-white gene substitution according to Vieillard et al. (2016) using the following primers: RNA guide oligonucleotide 1 sense 5′-CTTCGCTTTAGGCACCTCCAGG-3′, RNA guide oligonucleotide 1 antisense 5′-AAACCTGTGAGTGTCCTAAATTG-3′, RNA guide oligonucleotide 2 sense 5′-CTTGGGAGTACGAGTATCCGAC-3′, and RNA guide oligonucleotide 2 antisense 5′-AAA CGTGGGATACGTGCTGTC-3′. The upstream homology arm primers contained an EcoRI and a Apg918I site (underlined), respectively: 5′-GGGGAATCTTCTTATTCGCTCTGTTG-3′ and 5′-GATCGAGGCTGCTCATTAAAGCTG-3′. The downstream homology arm primers contained a SpeI and a Xhol site, respectively: 5′-CCCTGGAGATTGAAGCGCTACCTA-3′ and 5′-CCCTCGAGATTGAAGCGCTACCTA-3′.

**Online supplemental material**
Fig. S1 shows CoIP of Wdr92 with R2TP subunits, supplemental to Fig. 4. Table S1 shows a list of ciliary motility proteins in *Drosophila*. Table S2 shows abundance changes in motile cilia proteins in Wdr92 mutant testes: label-free quantitation intensities. Table S3 shows characteristics of R2TP/prefoldin-like genes in *Drosophila*. Table S4 shows RNAi lines and primers used in this study.

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The authors declare no competing financial interests.

Author contributions: P. zur Lage designed and conducted most of the experiments. P. Stefanopoulou carried out climbing and fertility assays for several genes. K. Styczynska-Soczka generated Wdr92-mVenus flies. N. Quinn and A. von Kriegsheim conducted the MS analyses. G. Mali and P. Mill contributed to experimental design, data interpretation, and writing. A.P. Jarman designed the experiments, conducted some experiments, and drafted the manuscript.

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