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# Cytoplasmic carboxypeptidase 5 regulates tubulin glutamylation and zebrafish cilia formation and function

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**ABSTRACT** Glutamylation is a functionally important tubulin posttranslational modification enriched on stable microtubules of neuronal axons, mitotic spindles, centrioles, and cilia. In vertebrates, balanced activities of tubulin glutamyl ligase and cytoplasmic carboxypeptidase deglutamylase enzymes maintain organelle- and cell type-specific tubulin glutamylation patterns. Tubulin glutamylation in cilia is regulated via restricted subcellular localization or expression of tubulin glutamyl ligases (*ttl*s) and nonenzymatic proteins, including the zebrafish TPR repeat protein *Fleer/Ift70*. Here we analyze the expression patterns of *ccp* deglutamylase genes during zebrafish development and the effects of *ccp* gene knockdown on cilia formation, morphology, and tubulin glutamylation. The deglutamylases *ccp2*, *ccp5*, and *ccp6* are expressed in ciliated cells, whereas *ccp1* expression is restricted to the nervous system. Only *ccp5* knockdown increases cilia tubulin glutamylation, induces ciliopathy phenotypes, including axis curvature, hydrocephalus, and pronephric cysts, and disrupts multicilia motility, suggesting that *Ccp5* is the principal tubulin deglutamylase that maintains functional levels of cilia tubulin glutamylation. The ability of *ccp5* knockdown to restore cilia tubulin glutamylation in *fleer/ift70* mutants and rescue pronephric multicilia formation in both *fleer*- and *ift88*-deficient zebrafish indicates that tubulin glutamylation is a key driver of ciliogenesis.

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## INTRODUCTION

Glutamylation is an evolutionarily conserved posttranslational modification that occurs prominently on  $\alpha$ - and  $\beta$ -tubulin associated with stable microtubules of mitotic spindles, neuronal axons, centrioles, and cilia (Edde *et al.*, 1990). Functionally, tubulin glutamylation regulates cytoskeletal processes by modulating microtubule-protein

interactions, such as those with neuronal kinesin motor KIF-1A during neurite extension (Ikegami *et al.*, 2007) and with septins during polarized vesicle transport (Spiliotis *et al.*, 2008). In addition, glutamylation stimulates the activity of the microtubule-severing protein spastin during microtubule disassembly (Lacroix *et al.*, 2010) and modulates the activities of inner arm dyneins to regulate cilia motility (Kubo *et al.*, 2010; Suryavanshi *et al.*, 2010). Variations in tubulin glutamylation patterns contribute to the structural and functional heterogeneity of microtubules in distinct organelles of vertebrate organisms (Verhey and Gaertig, 2007). Cytoplasmic microtubules are not glutamylated (Bobiniec *et al.*, 1998), whereas cilia transition zone microtubules are monoglutamylated, and neuronal and axonemal microtubules are polyglutamylated (Edde *et al.*, 1991; Fouquet *et al.*, 1994; Huitorel *et al.*, 2002; Kann *et al.*, 2003). Biochemically, glutamylation is a reversible process. Glutamate side chains are linked to genetically encoded glutamate(s) within C-termini of  $\alpha$ - or  $\beta$ -tubulin by tubulin glutamyl ligase members of the tubulin tyrosine ligase-like (TTL) protein family (Janke *et al.*, 2005; van Dijk *et al.*, 2007). Conversely, side chain glutamates are removed by tubulin deglutamylase members of the cytoplasmic carboxypeptidase (CCP) protein family (Rogowski *et al.*, 2010). It is believed that

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N.P., A.V., and I.A.D. designed the experiments; N.P. performed the experiments, with the exception of electron microscopy, which was performed by C.A.A.

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Abbreviations used: CCP, cytoplasmic carboxypeptidase; TTL, tubulin tyrosine ligase like.

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the diversity of microtubule subtypes generated by tubulin post-translational modifications is maintained by balanced activities of TLL glutamylases and CCP deglutamylases; however, the functional repertoire of *tll* and *ccp* genes in different organisms is not fully known.

The protein localization and expression patterns of vertebrate tubulin glutamyl ligases and deglutamylases suggest unique roles for different *tll* or *ccp* gene family members (Pathak *et al.*, 2011; Bosch Grau *et al.*, 2013; Lyons *et al.*, 2013). Green fluorescent protein (GFP) fusion proteins of TLL1, TLL9, and TLL11 show preferential localization to cilia basal bodies, whereas those of TLL5, TLL6, and TLL7 localize along ciliary axonemes (Janke *et al.*, 2005; van Dijk *et al.*, 2007). Functional studies reveal that TLL1 is required for respiratory cilia and sperm motility (Ikegami *et al.*, 2010; Vogel *et al.*, 2010), whereas TLL5 is exclusively required for sperm motility (Lee *et al.*, 2013). Analysis of *tll* mRNA expression in developing zebrafish and mouse brain ependymal cells suggests that *tll6* is uniquely important in ciliated cells, whereas *tll1*, *tll4*, and *tll7* may function in both ciliated cells and neurons (Pathak *et al.*, 2011; Bosch Grau *et al.*, 2013). *tll6* knockdown in zebrafish embryos or cultured ependymal cells primarily affects cilia motility (Pathak *et al.*, 2011; Bosch Grau *et al.*, 2013), whereas *tll1* is required for glutamylation of zebrafish secondary motor neurons (Pathak *et al.*, 2011) and TLL7 is required for growth of MAP2-positive neurites in PC12 cells (Ikegami *et al.*, 2006).

Tubulin deglutamylases also play important roles in cilia and neurons. In *Caenorhabditis elegans*, GFP fusion proteins of the tubulin deglutamylases CCP-1 and CCP-6 localize to amphid cilia (Kimura *et al.*, 2010; O'Hagan *et al.*, 2011). Mutation in *C. elegans* *ccp-1* causes excessive accumulation of KLP-6 kinesin and polycystin-2 in cilia and also increases the transport rate of OSM-3/KIF17 on axonemal microtubules (Kimura *et al.*, 2010; O'Hagan *et al.*, 2011). Deficiency of the mouse CCP1 tubulin deglutamylase increases polyglutamylation of brain neurons in *pcd* mice, leading to their progressive degeneration (Fernandez-Gonzalez *et al.*, 2002; Rogowski *et al.*, 2010). In zebrafish, whole-embryo quantitative PCR studies show that *ccp1*, *ccp2*, *ccp5*, and *ccp6* mRNA expression increases over the first 8 d of development; however, the tissue-specific expression of zebrafish *ccp* genes and how expression correlates with *ccp* gene function have not been fully explored (Lyons *et al.*, 2013).

In addition to changes in glutamylase and deglutamylase activity, tubulin glutamylation can be altered by mutations in putative basal body and intraflagellar transport (IFT)-associated proteins. Mutation in human *CEP41* results in loss of cilia, aberrant TLL6 localization, and consequent reduction in axonemal tubulin glutamylation (Lee *et al.*, 2012). Mutation in the zebrafish *fleer/ift70* gene causes significant reduction in axonemal tubulin glutamylation and loss of multiciliated cell ciliogenesis (Pathak *et al.*, 2007). *Fleer/ift70* is a tetratricopeptide repeat (TPR)-rich protein related to CeDYF-1/Crft-70 and is a component of the IFT complex (Fan *et al.*, 2010). It is not known whether *fleer* mutants lack tubulin glutamyl ligase activity or mislocalize Tll enzymes, or, alternatively, whether the *Fleer* protein limits the activity of deglutamylases or restricts their access to the cilia compartment.

Here we focus on the expression and function of zebrafish *ccp* deglutamylases in normal and *fleer*- or *ift88*-deficient cilia. We find that expression of zebrafish *ccp* deglutamylase genes *ccp2*, *ccp5*, and *ccp6* is strongly enriched in ciliated cell types, whereas *ccp1* expression is restricted to the nervous system and somites. Despite overlapping expression patterns, we find that *ccp5* is the major deglutamylase in zebrafish cilia and that *ccp5* deficiency can restore

cilia glutamylation in the *fleer* mutant and improve multiciliogenesis in *fleer*- and *ift88*-deficient zebrafish.

## RESULTS

### Expression of tubulin deglutamylases during zebrafish development

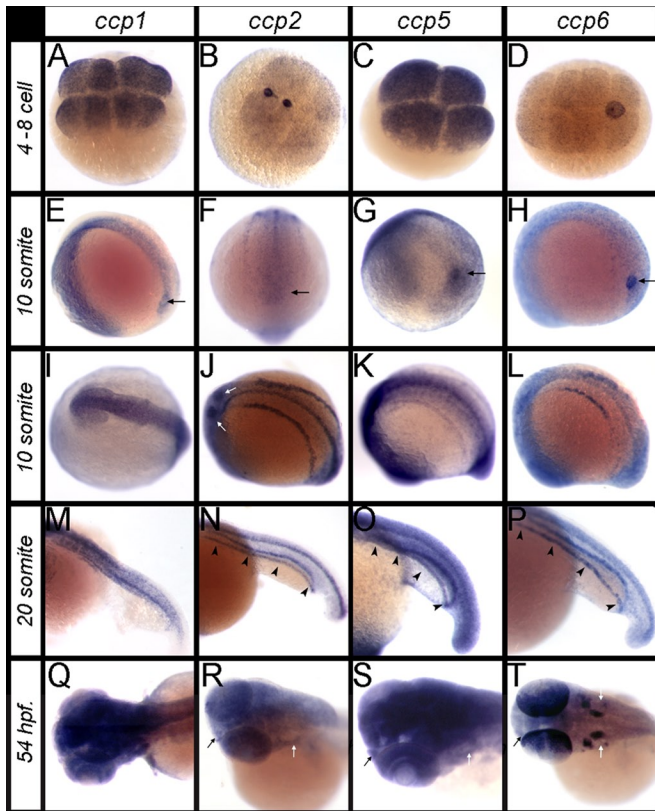
Tubulin deglutamylases are members of the M14 CCP protein family, alternatively known as ATP/GTP-binding protein-like family. Zebrafish harbor four orthologues of mouse deglutamylase genes: *Ccp1*, *Ccp2*, *Ccp5*, and *Ccp6* (Supplemental Figure S3A; Lyons *et al.*, 2013). A limited analysis of zebrafish *ccp* gene expression found evidence for specific expression of *ccp1*, *ccp2*, and *ccp5* in the pronephros and olfactory placode (Lyons *et al.*, 2013). We undertook a complete expression analysis of *ccp1*, *ccp2*, *ccp5*, and *ccp6* and found that *ccp1* expression was confined mainly to the developing CNS and somites at all developmental stages, whereas *ccp2*, *ccp5*, and *ccp6* were highly expressed in multiple ciliated cell types, including Kupffer's vesicle (KV), nephrogenic mesoderm, the pronephros, otic placode, olfactory placode, and lateral line organs (Figure 1 and Supplemental Figure S1). *ccp1* and *ccp5* were maternally expressed in four- to eight-cell-stage embryos (Figure 1, A and C). At the 10-somite stage, *ccp1* was expressed on the periphery of KV and in axial neurogenic cells (Figure 1, E and I), whereas *ccp5* and *ccp6* (Figure 1, G and H) but not *ccp2* (Figure 1F) were expressed in KV epithelial cells. Expression of *ccp2*, *ccp5*, and *ccp6* was also observed in the nephrogenic mesoderm at this stage and persisted until 54 h postfertilization (hpf) (Figure 1, J–L and N–P, and Supplemental Figure S1, F–H and J–L), whereas *ccp1* expression continued to be restricted to brain and spinal cord (Figure 1, M and Q). At 54 hpf, *ccp2*, *ccp5*, and *ccp6* were all expressed in the olfactory placode (Figure 1, R–T), while *ccp6* was also expressed in the retina and otic placode (Figure 1T). Overall the expression patterns of *ccp2*, *ccp5*, and *ccp6* show enrichment in ciliated cells, similar to the glutamyl ligase gene *tll6* and the glutamylation regulator *fleer* (Pathak *et al.*, 2007, 2011).

### *ccp5* knockdown induces ciliopathy phenotypes

To assess the function of *ccp* genes in ciliogenesis, we knocked down *ccp1*, *ccp2*, *ccp5*, and *ccp6* using antisense morpholinos. Injections of splice-blocking morpholino oligos caused near-complete elimination of wild-type *ccp* gene mRNA (Figure 2A and Supplemental Figure S2A), resulting in truncated splice products predicted to encode proteins lacking carboxypeptidase domains (Supplemental Figure S3, B–F). Of these four genes, only *ccp5* knockdown induced a typical spectrum of ciliopathy phenotypes, including axis curvature, pronephric cysts, and hydrocephalus (Figure 2B). Whereas isolated hydrocephalus was observed in *ccp1* morphants (Figure 2, B and C), this was accounted for by widespread cell death in the CNS (Supplemental Figure S2B), similar to the CCP1-deficient mouse *pcd* mutant phenotype (Mullen *et al.*, 1976; Rogowski *et al.*, 2010), with subsequent obstruction of the spinal canal with cell debris (Supplemental Figure S2B). Although knockdown of *ccp2* eliminated all wild-type *ccp2* mRNA, no obvious embryonic phenotype was frequently observed (Supplemental Figure S2, A and B).

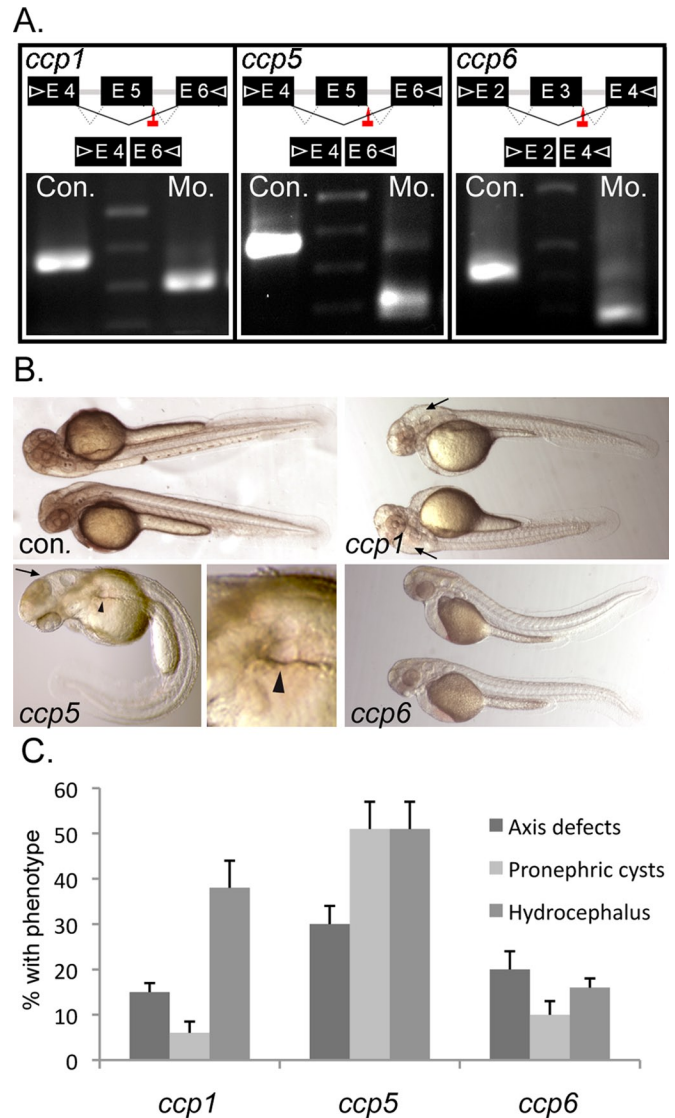
### *ccp5* deficiency induces cilia microtubule hyperglutamylation and motility defects

To determine the cellular basis of ciliopathy phenotypes in *ccp5*-deficient embryos, we analyzed cilia in the pronephros and brain ventricles with respect to glutamylated tubulin content, length, and motility. Double immunolabeling of whole-mount embryos with monoclonal antibody (mAb) GT335 (recognizing monoglutamylated



**FIGURE 1:** RNA in situ hybridization patterns of *ccp1*, *ccp2*, *ccp5*, and *ccp6* during zebrafish development. (A–D) The four- to eight-cell-stage embryos, showing maternal transcripts of (A) *ccp1*, (B) *ccp2*, (C) *ccp5*, and (D) *ccp6*. (E–H) Lateral and caudal views of 10-somite embryos, showing expression of (E) *ccp1*, (F) *ccp2*, (G) *ccp5*, and (H) *ccp6* relative to the Kupffer's vesicle (black arrows). (I–L) Dorsal views of 10-somite embryos, showing expression of (I) *ccp1* in the CNS and (J) *ccp2*, (K) *ccp5*, and (L) *ccp6* in the spinal canal and bilateral nephrogenic mesoderm. (M–P) Twenty-somite embryos, showing that (M) *ccp1* is expressed medially in somites and spinal canal, whereas (N) *ccp2*, (O) *ccp5*, and (P) *ccp6* expression is prominent medially in the spinal canal and bilaterally in the pronephroi (black arrowheads). (Q–T) Dorsal views of the head in 54-hpf larvae, showing (Q) *ccp1*, (R) *ccp2*, (S) *ccp5*, and (T) *ccp6* are widely expressed in CNS, eye, otic placode (white arrows), and olfactory placode (black arrows). In T, note the distinct expression of *ccp6* in otic placodes and posterior cell layers of the eye.

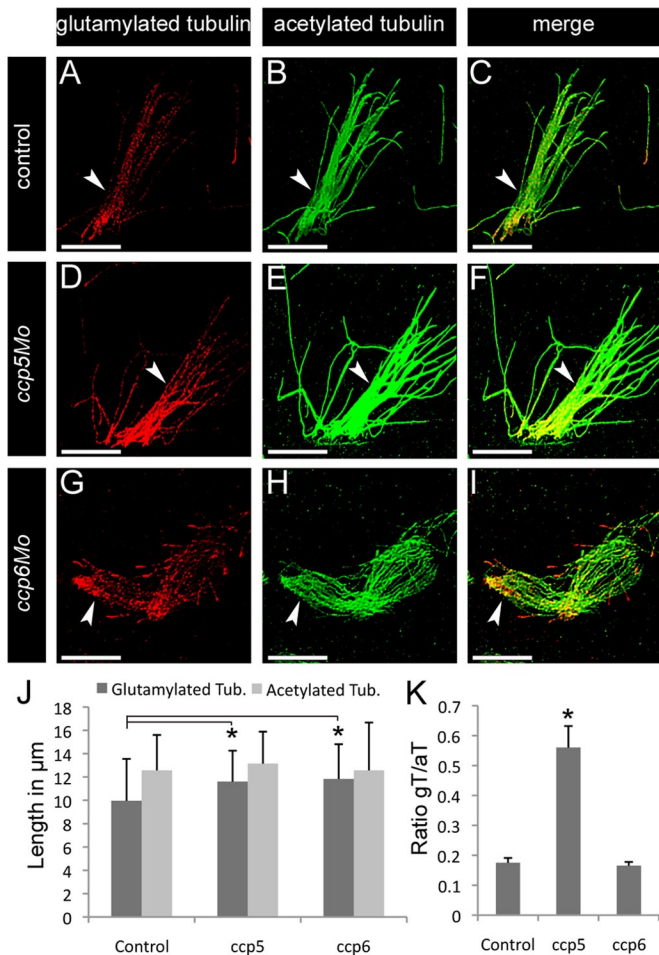
or polyglutamylated tubulin) and mAb 6-11B-1 (specific to acetylated tubulin) allowed direct assessment of cilia tubulin glutamylation in wild-type versus *ccp5*-deficient embryos (Figure 3). Tubulin glutamylation in control single cilia and multicilia extends along the length of the cilium (Figure 3, A and C, and Supplemental Figure S4, A and C; also see later discussions of Figures 5, A and C, and 6), decreasing from the base to the tip (Pathak *et al.*, 2007). Relative to control cilia imaged under identical conditions, glutamylated tubulin immunoreactivity was significantly increased relative to acetylated tubulin in *ccp5*-deficient cilia (Figure 3, D–F, and later discussions of Figures 5, D–F, and 6), whereas it appeared unchanged in cilia of *ccp1* (unpublished data) and *ccp6* morphants (Figure 3, G–I). Measurements of the axonemal extent of immunolabeling of single pronephric cilia for glutamylated versus acetylated tubulin revealed a small but significant increase in the extent of tubulin glutamylation in *ccp5*-deficient cilia (Figure 3J), whereas overall cilia length (acetylated tubulin) was not significantly different. To quantify tubulin



**FIGURE 2:** *ccp5* knockdown alone induces the typical spectrum of ciliopathy phenotypes in wild-type zebrafish. (A) Splice donor sites targeted by antisense morpholinos (red arrows) in *ccp1*, *ccp5*, and *ccp6* genes and agarose gel analysis showing RT-PCR amplicons of *ccp1*, *ccp5*, and *ccp6* generated using primer pairs (arrowheads) in indicated exons were smaller in morphant (Mo.) embryos than in respective control (Con.) In each case, the reduced size of morphant amplicon relative to control matched the size of the deleted target exon. (B) External morphology of 2.5-d-old zebrafish injected with optimal dose of control or antisense morpholinos designed to knock down *ccp1*, *ccp5*, and *ccp6* genes, respectively. (C) Frequency of phenotype associated with cilia defects. *ccp1* morphants mostly exhibit isolated hydrocephalus, whereas *ccp5* morphants exhibit hydrocephalus and pronephric cysts, often with acutely curved body axis. Replicates: *ccp1*×5Mo (*n* = 181 embryos/4 injections); *ccp5*×5Mo (*n* = 275 embryos/5 injections); *ccp6*×3Mo (*n* = 261 embryos/5 injections).

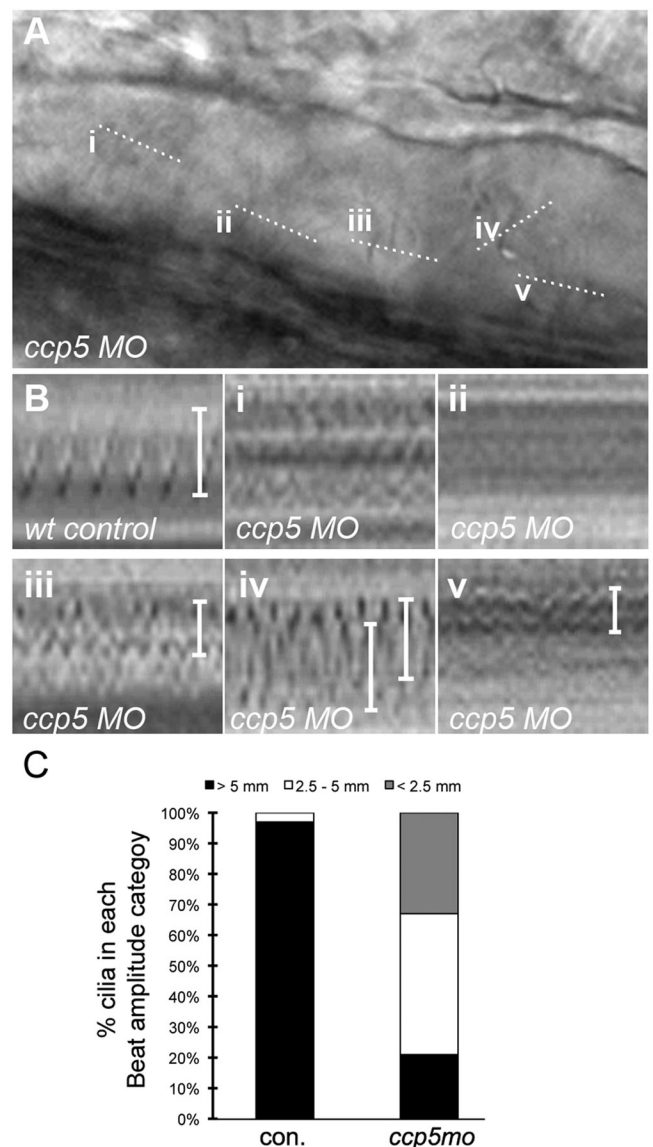
glutamylation in cilia, we acquired multiple images of immunolabeled multicilia under identical conditions and analyzed them using ImageJ to measure total pixel intensity for red (glutamylated tubulin) and green (acetylated tubulin) channels in the same region of interest. Expressing the intensity of the red and green channels as a ratio, we found that pronephric cilia tubulin glutamylation increased





**FIGURE 3:** *ccp5* knockdown induced hyperglutamylation of cilia microtubules. Representative images of pronephric cilia from 2.5-d-old zebrafish larvae double immunolabeled with glutamylated tubulin-specific mAb GT335 (red) and acetylated tubulin-specific mAb 6-11B-1 (green). Scale bars, 10 µm. (A–C) Pronephric multicilia (arrowheads) from control larva showing that (A) glutamylated tubulin normally decreases from the base to the tip of axonemes, in contrast to (B) uniformly distributed acetylated tubulin. (D–F) Pronephric multicilia from *ccp5* morphant larva showing (D) enhanced labeling intensity of glutamylated tubulin along their axonemes marked by (E) acetylated tubulin. (G–I) Pronephric multicilia from *ccp6* morphant, showing that (G) labeling intensity of glutamylated tubulin along their axonemes marked by (H) acetylated tubulin is similar to control. (J) Average length of glutamylated tubulin labeled segment increased in the pronephric cilia of *ccp5* and *ccp6* morphants relative to controls, although length of their acetylated tubulin-labeled segments was similar. Control ( $n = 33$ , cilia/4 larvae), *ccp5* morphants ( $n = 42$  cilia/4 larvae), *ccp6* morphants ( $n = 31$  cilia/4 larvae). Error bars, SD;  $t$  test,  $*p < 0.05$ . (K) Average intensity ratio of glutamylated tubulin relative to acetylated tubulin significantly increased in pronephric cilia of *ccp5* morphants relative to control or *ccp6* morphants.  $n = 36$  cilia/4 control larvae; 33 cilia/4 *ccp5* morphant larvae; 45 cilia/4 *ccp6* morphant larvae. Error bars, SEM;  $t$  test,  $*p < 0.05$ .

2.5-fold (0.56) in *ccp5*-deficient cilia compared with control (0.20; Figure 3K), whereas the average ratio of glutamylated tubulin to acetylated tubulin in *ccp6*-deficient cilia was similar to control (0.165). High-speed videomicroscopy analysis of pronephric multicilia motility from 2.5-d-old control larvae revealed a synchronous waveform with an average beat amplitude of 6 µm (Figure 4, B

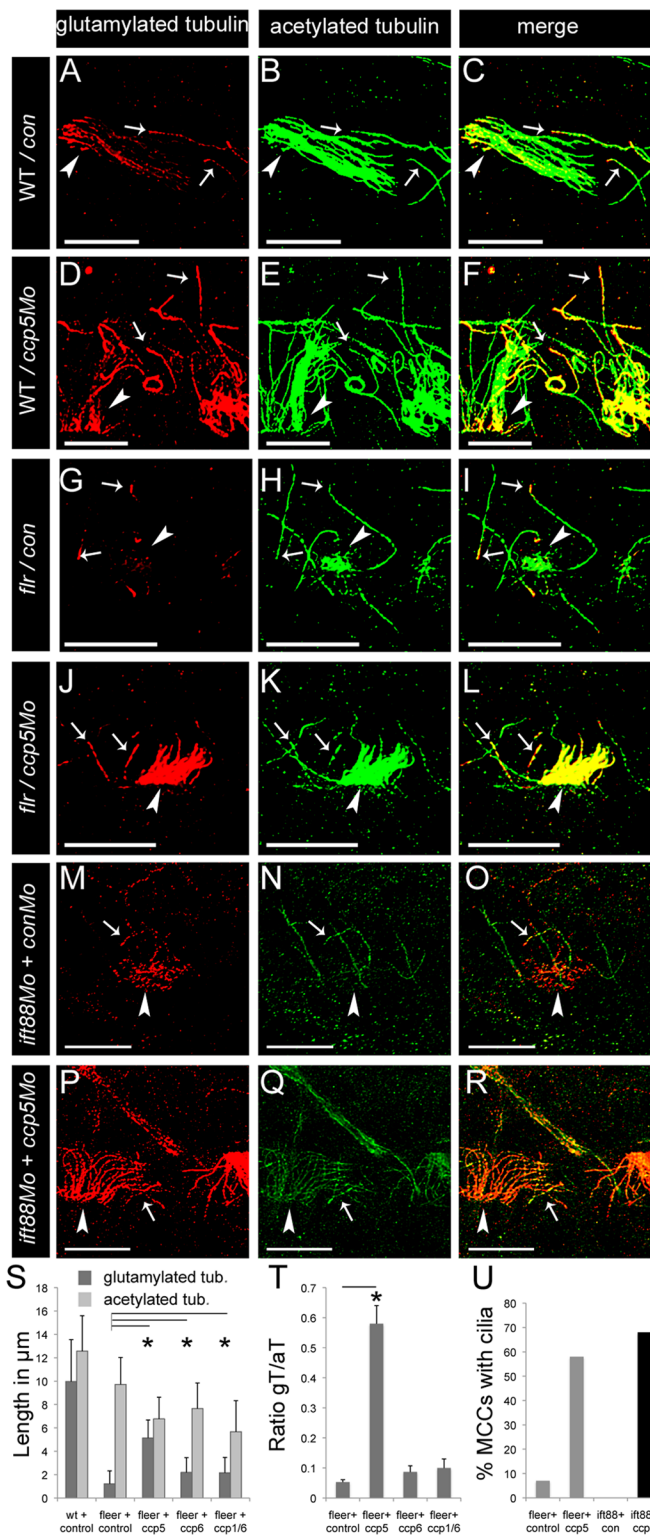


**FIGURE 4:** *ccp5* inactivation perturbs cilia motility. (A) Frame of a movie taken of pronephric cilia from a 2.5-d-old *ccp5* morphant. Lower-case Roman numerals indicate multicilia analyzed for motility defects; dotted lines indicate the plane of the line scanned through the cilium. (B) Kymograms of pronephric cilia from a wild-type control and *ccp5*-deficient multicilia (i–v) from the movie frame in A. (C) Quantitative representation of beat amplitude categories of pronephric multicilia observed in control embryos ( $n = 36$  cilia from eight embryos) and *ccp5* morphants ( $n = 33$  cilia from seven embryos).

and C, and Supplemental Movie S1). In contrast, motile multicilia in the *ccp5*-deficient morphants were uncoordinated with individual *ccp5*-deficient cilia either completely paralyzed or moving independently with significantly reduced beat amplitudes (Figure 4, B and C, and Supplemental Movie S2). Cilia tubulin hyperglutamylation thus appears to inhibit motility-associated functions of microtubules.

#### **ccp5 knockdown restores tubulin glutamylation and promotes multicilia assembly in IFT-deficient zebrafish**

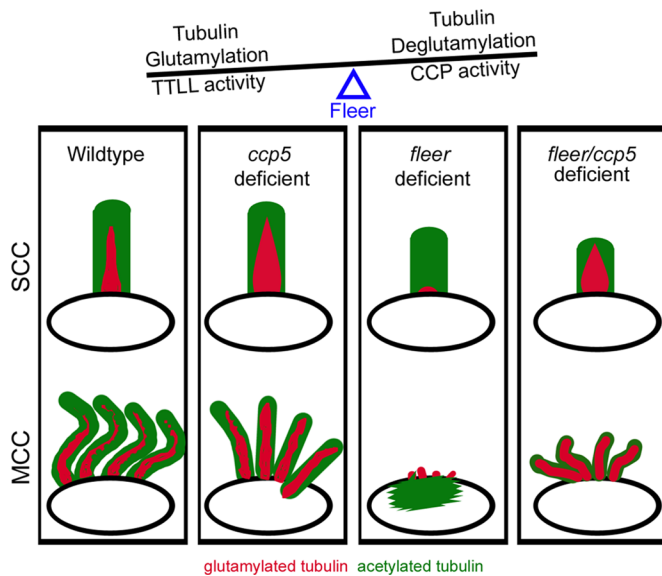
We previously showed that mutation in the zebrafish cilia TPR protein Fleeer/Ift70 significantly reduced axonemal tubulin



**FIGURE 5:** *ccp5* knockdown in *Fleer*- and *Ift88*-deficient zebrafish promotes pronephric multicilia assembly. (A–R) Representative images of pronephric single cilia (white arrows) and multicilia (white arrowheads) of 2.5-d-old zebrafish larvae double immunolabeled with glutamylated tubulin–specific mAb GT335 (red) and acetylated tubulin–specific mAb 6-11B-1 (green). Scale bars, 10 µm. (A–C) Pronephric cilia in control larva showing (A) that glutamylated tubulin levels gradually decrease from base to tip of axonemes; (B) that acetylated tubulin is uniformly distributed along the entire length of axonemes; and (C) their merge. (D–F) Pronephric cilia in *ccp5*

glutamylated tubulin, cilia motility, and single cilia length and blocked ciliogenesis in multiciliated cells (Pathak *et al.*, 2007). Given the increase in cilia glutamylation we observed with *ccp5* knockdown, we tested whether compound deficiency of *ccp* deglutamylases and *fleer* would restore glutamylation in *fleer* mutant cilia. We injected *fleer<sup>ca1</sup>* in-cross clutches with control, *ccp5*, *ccp6*, and *ccp1/ccp6* morpholinos and identified homozygous *fleer<sup>ca1</sup>* 2.5-d-old larvae by genotyping. We then performed double immunolabeling with mAb GT335, specific to glutamylated tubulin, and 6-11B-1, specific to acetylated tubulin. Analysis of glutamylated tubulin in control (Figure 5A) and *fleer* mutant pronephric single cilia (arrows in Figure 5, G and I; Figure 6) confirmed that tubulin glutamylation was significantly reduced and restricted to the cilia base in *fleer* larvae. Multiciliated cells in the *fleer* mutants do not extend axonemes or show glutamylated basal bodies (arrowhead, Figure 5G; Pathak *et al.*, 2007) but instead have aberrant apical cytoplasmic accumulation of acetylated tubulin (arrowhead, Figure 5H; Supplemental Figure S4, E, H, and K, and Figure 6). Strikingly, compound *fleer* mutant/*ccp5* morphants showed significantly increased intensity of glutamylated tubulin reactivity in single cilia and prominent glutamylated tubulin-positive axonemes extending from pronephric multiciliated cells (Figures 5, J–L, and 6). Restoration of glutamylation or multicilia axoneme elongation was not observed in compound *fleer/ccp6* morphants (Supplemental Figure S5, G–I) or *fleer/ccp1/ccp6* morphants

morphant showing (D) glutamylated tubulin at elevated levels along the entire length of axonemes, (E) acetylated tubulin, and (F) their merge. (G–I) Pronephric cilia of *fleer* mutant injected with control morpholino showing (G) glutamylated tubulin restricted near the base of single cilia and absent in multicilia, (H) acetylated tubulin in axonemes of single cilia and accumulated in cytoplasm of multiciliated cells, and (I) their merge. (J–L) Pronephric cilia of *fleer* mutant injected with *ccp5*×5Mo showing (J) glutamylated tubulin at elevated levels in axonemes of both single and multicilia, (K) acetylated tubulin in axonemes of single cilia and restored multicilia (note reduced cytoplasmic accumulation of acetylated tubulin in multiciliated cells), and (L) their merge. (M–O) Pronephric cilia of *ift88* morphants showing (M) glutamylated tubulin gradually decreasing along single cilia and abnormally accumulated at the base of multicilia, (N) acetylated tubulin present along single cilia but notably absent in multiciliated cells, and (O) their merge. (P–R) Pronephric cilia in double morphant of *ift88* and *ccp5* showing (P) glutamylated tubulin at elevated levels in axonemes of both single and restored multicilia (note reduced cytoplasmic accumulation of glutamylated tubulin in multiciliated cells), (Q) acetylated tubulin in single and restored multicilia, and (R) their merge. (S) Average length of glutamylated tubulin– and acetylated tubulin–labeled segments in pronephric cilia of WT zebrafish injected with control Mo ( $n = 33$  cilia/4 larvae), *fleer* mutants injected with control Mo ( $n = 50$  cilia/4 larvae), *ccp5*×5Mo ( $n = 55$  cilia/5 larvae), *ccp6*×3Mo alone ( $n = 60$  cilia/4 larvae), and the combination *ccp1*×5Mo/*ccp6*×3Mo ( $n = 86$  cilia/5 larvae). Error bars, SD; t test, \* $p < 0.05$ . (T) Average intensity ratios of glutamylated tubulin relative to acetylated tubulin measured in individual double-immunolabeled pronephric single cilia and multicilia from *fleer* mutant larvae injected with control MO ( $n = 48$  cilia/4 larvae), *ccp5*×5Mo ( $n = 78$  cilia/6 larvae), *ccp6*×3Mo alone ( $n = 41$  cilia/4 larvae), and the combination *ccp1*×5Mo/*ccp6*×3Mo ( $n = 36$  cilia/4 larvae). Error bar, SEM; t test, \* $p < 0.05$ . (U) Percentage of MCCs that extend axonemes >2 µm from their apical surface in the pronephros of *fleer* mutants injected with control morpholino ( $n = 55$  MCCs/6 larvae), *fleer* mutants injected with *ccp5*×5Mo ( $n = 48$  MCCs/8 larvae), zebrafish injected with the combination *ift88*Mo and control Mo ( $n = 43$  MCCs/8 larvae), and zebrafish injected with the combination *ift88*Mo and *ccp5*×5Mo ( $n = 41$  MCCs/8 larvae).



**FIGURE 6:** Schematic depicting *ccp5* knockdown-induced changes to tubulin glutamylation in normal and *flee*-deficient cilia. In normal cilia of wild-type single ciliated cells (SCCs) and MCCs, glutamylated tubulin is enriched maximally at the base and gradually decreases toward the tip. Loss of *ccp5* in otherwise normal cilia significantly increases the overall amount and extent of glutamylated tubulin without increasing cilia length. Hyperglutamylation inhibits efficient cilia motility. Glutamylated tubulin is significantly reduced and restricted to the base of short, single cilia that persist in the *flee* mutants. *Flee*-deficient cilia remain competent for tubulin glutamylation and show greater-than-normal increase in overall intensity and extent when *Ccp5* deglutamylase is knocked down in the *flee* mutants. Multicilia in *flee* mutants are more sensitive to the loss of glutamylation; however, multiciliated cells in compound *flee*/*ccp5*-deficient zebrafish show improved assembly of cilia upon increase in tubulin glutamylation.

(Supplemental Figure S5, J–L). Quantification of the extent of glutamylated and acetylated tubulin in individual cilia from *flee* mutant or compound *flee*/*ccp5*-gene deficient larvae (Supplemental Figure S5) revealed that whereas glutamylated tubulin was confined to an average 1.2  $\mu\text{m}$  length at the base of single cilia in *flee* mutants, this increased significantly to 5.1  $\mu\text{m}$  in cilia of *flee*/*ccp5*-deficient larvae. However, the extent of acetylated tubulin in single pronephric cilia of *ccp5*/*flee*-deficient larvae (6.8  $\mu\text{m}$ ) was reduced compared with cilia deficient in *flee* alone (9.7  $\mu\text{m}$ ). Compound *flee*/*ccp6*- and *flee*/*ccp1/ccp6*-deficient larvae did not show a significant restoration of the axonemal extent of glutamylated tubulin reactivity (2.2  $\mu\text{m}$ ; Supplemental Figure S5). Assessment of the overall pixel intensity of glutamylated tubulin reactivity relative to acetylated tubulin in the same cilia (Figure 5T) revealed a low intensity ratio for glutamylated:acetylated tubulin in pronephric single cilia of *flee* (0.05), *flee*/*ccp6*-deficient (0.08), and *flee*/*ccp1/ccp6*-deficient larvae (0.1) compared with control cilia (0.20). Cilia of *flee*/*ccp5*-deficient larvae showed a remarkable recovery in the glutamylated:acetylated tubulin ratio intensity (0.58; Figure 5T). Recovery of tubulin glutamylation in compound *flee*/*ccp5* deficient multiciliated cells (MCCs) correlated with re-appearance of short apical MCC cilia (Figures 5, J–L and U, and 6), indicating that *ccp5* knockdown could partially rescue *flee* mutant multiciliogenesis.

To test whether rescue of multiciliogenesis by *ccp5* knockdown was specific to *flee* mutants, we examined *ift88*-deficient zebrafish

that similarly fail to generate cilia on MCCs (Tsujiyama and Malicki, 2004; Kramer-Zucker *et al.*, 2005). In 2.5-d-old *ift88* morphants, pronephric MCCs show glutamylated basal bodies (Figure 5M and Supplemental Figure S5A) but no acetylated tubulin (Figure 5, N and O) or Arl13b-positive cilia (Supplemental Figure S5, B and C). In contrast, in *ift88/ccp5* double morphants, ~70% of pronephric MCCs (Figure 5U) showed axonemes that were strongly labeled by glutamylated tubulin (arrowheads in Figure 5P; Supplemental Figure S5D), acetylated tubulin (Figure 5, Q and R), or Arl13b (Supplemental Figure S5, E and F). Our results demonstrate that cilia in the *flee* mutant are competent for tubulin glutamylation (Figure 6) and suggest that high levels of tubulin glutamylation generated by *ccp5* knockdown can exert a dominant positive effect on multiciliogenesis, even in cells deficient for *Fleer/Ift70* or *Ift88*.

## DISCUSSION

Tubulin glutamylation is an important posttranslational modification that is regulated by the balanced activities of *tll* tubulin ligases and *ccp* tubulin deglutamylases (Janke and Bulinski, 2011). Our results show that the zebrafish *ccp* gene family is broadly expressed during development and *Ccp5* is the key deglutamylase that maintains the functional set-point of cilia tubulin glutamylation. Tubulin glutamylation is also regulated in cilia by the *Fleer/Ift70* protein, and we show that *ccp5* knockdown partially overcomes multicilia elongation defects in *Fleer/Ift70*- and *Ift88*-deficient zebrafish. Our findings suggest that increased glutamylation can drive initial assembly of multicilia axonemes but that prevention of excessive tubulin glutamylation by *ccp5* deglutamylase activity is essential for mature cilia motility and function.

### Zebrafish *ccp* gene expression and function

Our analysis of *ccp* gene expression in zebrafish revealed that all four zebrafish *ccp* genes were expressed throughout embryonic development, extending a previous analysis of *ccp* gene expression in zebrafish (Lyons *et al.*, 2013). Specifically, we find that in addition to *ccp1* and *ccp5*, the tubulin deglutamylase genes *ccp2* and *ccp6* are expressed in multiple ciliated tissues throughout embryogenesis. Although overlapping expression of *ccp2*, *ccp5*, and *ccp6* suggested the possibility of redundant deglutamylating activity in cilia, we found that *ccp5* was the principal enzyme involved in regulating ciliary glutamyl tubulin levels. Previously *ccp5* knockdown was shown by Western blotting to globally increase embryo glutamyl tubulin, but this did not distinguish between cilia and the abundant tubulin glutamylation in brain and spinal cord neurons (Lyons *et al.*, 2013). In addition, in contrast to previous reports, we did not see an increase in cilia tubulin glutamylation in *ccp1*-deficient embryos (Lyons *et al.*, 2013). Although this lack of effect could reflect structural defects of the axoneme similar to those seen in the *C. elegans* *ccpp-1* mutants (O'Hagan *et al.*, 2011), this could simply be due to restricted expression of *ccp1* in the zebrafish nervous system. In addition, the reported increase in glutamyl tubulin immunoreactivity in the *ccp1*-knockdown pronephric duct was restricted to the cytoplasm and not cilia associated (Lyons *et al.*, 2013). In *ccp1*-deficient embryos we did observe marked hydrocephalus, a phenotype often associated with ependymal cilia paralysis and impaired cerebrospinal fluid flow (Ibanez-Tallon *et al.*, 2004; Kramer-Zucker *et al.*, 2005). However, because the mouse *Ccp1/Pcd* mutant is known to exhibit a neuronal cell death phenotype, we used the apoptosis reporter annexin-GFP zebrafish transgenic (van Ham *et al.*, 2010) to examine the possibility that cell debris from dying neurons might obstruct the spinal canal and found that this was indeed the case. This result is a cautionary note in interpreting hydrocephalus purely as a ciliopathy,



since any physical obstruction of the spinal canal may produce hydrocephalus. Of the four *ccp* genes in zebrafish, we conclude that *ccp5* is specifically required to regulate axonemal tubulin glutamylation.

### ccp-dependent regulation of cilia structure and function

Our work suggests that Ccp5 is normally active in cilia and is important for maintaining the appropriate level of axonemal tubulin glutamylation, since *ccp5* knockdown increased cilia tubulin glutamylation. Ccp5 may normally play a role in preventing formation of supernumerary glutamate chains that could compromise motility-associated functions of cilia microtubules. Hyperglutamylation has been shown to arrest motility of *Tetrahymena* cilia (Janke et al., 2005) and induce structural defects in axonemal microtubules (O'Hagan et al., 2011). Our results show that loss of *ccp5* induces hyperglutamylation in zebrafish pronephric cilia and reduces cilia beat coordination and beat amplitude without affecting beat frequency. This may reflect a preferential effect of hyperglutamylation on inner dynein arm activity, consistent with previously reported effects of TTL deficiency on axonemal microtubule sliding and the role of inner dynein arms in regulating cilia waveform (Brokaw and Kamiya, 1987; Wood et al., 2007; Suryavanshi et al., 2010). We also observed microtubule doublet B-tubule gap defects, similar to *tll* loss-of-function cilia (Pathak et al., 2011), in a minority of cilia examined in *ccp5* morphants (unpublished data). Because cilia were uniformly hyperglutamylated but only a minority showed ultrastructural defects, it is likely the B-subfiber gaps are not a primary effect of hyperglutamylation but instead represent a late-stage breakdown of cilia structural integrity. Unlike mutations that completely disrupt cilia structure and show fully penetrant kidney cyst formation (Sun et al., 2004; Sullivan-Brown et al., 2008; Huang and Schier, 2009), kidney cyst formation in *ccp5*-knockdown embryos was only partially penetrant. This may reflect the fact that kidney cilia motility was not completely disrupted by *ccp5* knockdown but instead was disorganized and reduced in beat amplitude. We observed similar partial penetrance of kidney cyst formation in *tll3/6*-gene knockdowns, which reduce tubulin glycylation and glutamylation (Pathak et al., 2011) and produce a similar disorganized cilia beat pattern. Overall the results indicate that hypoglutamylation or hyperglutamylation of cilia tubulin disrupts cilia function and suggests that *tll* ligases and *ccp* glutamylases act in concert to establish a functional "set-point" for tubulin modifications.

### Ccp5, tubulin hyperglutamylation, and IFT proteins in cilia

The restoration of axonemal glutamylation and multicilia formation by *ccp5* knockdown in IFT protein-deficient zebrafish highlights the idea that tubulin glutamylation can have a dominant, positive effect on ciliogenesis. We chose to examine the effect of *ccp5* knockdown and hyperglutamylation on the *fleer*-mutant phenotype because we showed previously that zebrafish *fleer*-mutant single cilia lack tubulin glutamylation and that *fleer*-mutant multicilia fail to form (Pathak et al., 2007). Because multiciliated cells contain more cilia (>20), it may be that the more severe ciliogenesis defect in these cells is due to more stringent IFT protein stoichiometric requirements for ciliogenesis. Although the exact mechanism of Fleer/Ift70 function in tubulin glutamylation is not known, there are several possibilities to account for loss of glutamylation in *fleer* mutants. Fleer may act to maintain the structural integrity of B-tubule protofilaments, the substrate for microtubule glutamylation (Kann et al., 1995; Multigner et al., 1996; Lechtreck and Geimer, 2000). Alternatively, it may serve as an IFT adaptor protein to facilitate axonemal transport of Tll glutamylases and other essential axonemal

proteins (Pathak et al., 2007; Fan et al., 2010; Taschner et al., 2011, 2012; Zhao and Malicki, 2011). A new hypothesis based on our present work is that Fleer may act to suppress the activity of tubulin deglutamylases like Ccp5. Our finding that *fleer/ift70*-mutant cilia remain competent for glutamylation in *ccp5*-deficient embryos, as well as the finding that *C. elegans ccpp-1*-mutant cilia show enhanced glutamylation that is not reduced by mutation in the *fleer/ift70* orthologue Dyf-1 (O'Hagan et al., 2011), supports this idea. Whatever the mechanism of Fleer activity may be, our results show a dominant effect of tubulin hyperglutamylation on multiciliogenesis not only in *fleer/ift70* mutants, but also in *ift88* morphants. Given that some maternal *fleer* mRNA may persist in zygotic mutants and that morpholino knockdown of *ift88* may not be complete, it is possible that some Fleer and Ift88 protein may be present and interact with higher affinity for hyperglutamylated axonemes. Nonetheless, our results raise the possibility that in some contexts, deficiencies of individual IFT proteins may be bypassed in ciliogenesis by enhanced recruitment of compensatory IFT proteins to forming cilia axonemes or possibly by enhanced activity of microtubule motor proteins in IFT transport. The identification and characterization of Tll tubulin-modifying enzymes and Ccp deglutamylases should make it possible to determine the function of tubulin glutamylation in ciliogenesis.

## MATERIALS AND METHODS

### Zebrafish strains, maintenance, and genotyping

Wild-type TuAB, *fleer* mutant, transgenic CD-41-GFP, and transgenic UAS-annexin-V–yellow fluorescent protein lines were maintained according to standard procedures and used to obtain embryos for morpholino injections. Pigment formation in zebrafish larvae was suppressed by addition of (0.003%) 1-phenyl 2-thiourea to egg water after 20 and 24 hpf. The *fleer<sup>ca1</sup>* mutant allele encodes a truncated 186–amino acid Fleer N-terminal peptide as opposed to the full-length Fleer protein of 651 amino acids (Leshchiner et al., 2012). Embryos from in-crosses of *fleer<sup>ca1</sup>* heterozygotes were genotyped by polymorphism of a simple sequence repeat in the vicinity of the *fleer* locus on zebrafish chromosome 3. Genomic DNA from tail clips of embryos was used to obtain DNA amplicons using the following PCR primers:

nZ1071F, CAGCTGCTACAGCAACCTGA

nZ1071R, GGACGCGGTATGTAACCTGT

### Zebrafish *ccp* clones

Full- and partial-length cDNAs of zebrafish *ccp1* were amplified by reverse transcription (RT)-PCR based on their predicted sequence and cloned into pDONR 221 vector. Full-length clones of *ccp2* in pME18S-FL3 (BC146747) and *ccp5/agb15* in pExpress-1 (BC080248) and a partial *ccp6* EST (BC093361) in pME18S-FL3 were purchased from Open Biosystems (Huntsville, AL). Evolutionary analysis of *ccp* homologues is presented in Supplemental Figure S3 and was generated using clustalw (Chenna et al., 2003) and drawtree in the Phylip 3.695 software package (Felsenstein, 1997).

### Morpholino knockdown of *ccp* genes

Antisense morpholinos were designed to block specific splice donor sites within *ccp1*, *ccp2*, *ccp5*, and *ccp6* deglutamylase genes and purchased from Gene Tools (Philomath, OR). In the following morpholinos, the name of the targeted deglutamylase gene precedes the letter x, followed by the number of the specific target exon. The targeted splice site is underlined in the following sequences (5'–3') of morpholinos:



*Ccp1x5Mo*: TTAAGAACACCAAAACTCACCGACA  
*Ccp2x2Mo*: CCATACAGAAGTGGAGCTTACCTGA  
*Ccp5x5Mo*: TCCTCTTAATGTGCAGATACCCGTT  
*Ccp5x8Mo*: AAGTTTGACTCCTAGACGTACCTGT  
*Ccp6x3Mo*: TTTACGTCCAAGTGCTTACCTGAGT  
*ift88(polaris)AUGMo*: CTGGGACAAGATGCACATTCTCCAT  
(Kramer-Zucker et al., 2005)

Control injections were performed using a heterologous inverse sense morpholino against *foxJ1a*:

*foxJ1a conMo*: CGTCCATTGTGAAAAGTGTAAACCA  
(Hellman et al., 2010)

Morpholino oligonucleotides were dissolved in RNase-free water to a 2 mM stock concentration. For injections, morpholino dilutions were made in 100 mM KCl, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), and 0.1% phenol red. A Nanoliter 2000 microinjector (WPI Instruments, Sarasota, FL) was used to inject 4.6-nl volumes in two- to four- cell embryos. To detect morpholino-induced splicing defects upon injection of *ccp5x5mo* or *ccp5x8mo*, nested RT-PCR was performed on total RNA extracted from individual embryos using primers listed in Supplemental Table S1.

### RNA in situ hybridization

Linearized DNA templates of *ccp1* and *ccp5* containing T7 promoter sites were obtained by restriction digestion of their clones with *Xma1* and *EcoR1*, respectively. Linearized DNA templates of *ccp2* and *ccp6* cDNAs were generated from their pME18S-FL3 vectors by PCR, with the reverse primer engineered to contain the T7 promoter. Digoxigenin-labeled full-length antisense riboprobes of *ccp1*, *ccp2*, *ccp5*, and *ccp6* were synthesized by in vitro transcription using the T7 polymerase. Whole-mount RNA in situ hybridization was performed as described previously (Liu et al., 2007; Thisse and Thisse, 2008). For imaging, the stained embryos were cleared in dimethyl formamide and mounted in 80% glycerol. Images were obtained using a Spot image digital camera mounted on a Leitz MZ12 stereomicroscope (Leica, Wetzlar, Germany) or Nikon E800 microscope (Nikon, Melville, NY).

### Immunofluorescence: qualitative and quantitative analysis

Whole zebrafish larvae were fixed for immunolabeling in Dent's fixative (80% methanol, 20% dimethyl sulfoxide) at 4°C overnight. For visualization of pronephric cilia in control morphants, distension of the pronephric lumen was induced in the live larva by mechanical obstruction of their cloaca 1 h before fixation. The fixed specimens were rehydrated gradually and washed with phosphate-buffered saline (PBS) containing 0.5% Tween 20 (PBST) and blocked for 2 h with 5% normal goat serum before antibody labeling. For immunolabeling, the larvae were sequentially incubated with appropriate dilutions of each primary and secondary antibody overnight at 4°C. The dilutions of primary antibodies used were as follows: mAb GT335, 1:400 (Enzo Life Sciences, Plymouth Meeting, PA); mAb 6-11B-1, 1:800 (Sigma-Aldrich, St. Louis, MO); and Arl13b, 1:250 (gift from Z. Sun, Yale University). Immunoreactivity was detected using the secondary antibodies Alexa 546-conjugated goat anti rabbit (1:800), Alexa 546-conjugated goat anti-mouse, and Alexa 488-conjugated donkey anti-mouse (1:800). Immunolabeling with two mouse antibodies was performed as described earlier, with the first primary and secondary antibodies briefly fixed with 4% paraformaldehyde and their reactive sites blocked by sequential incubations in 10% unconjugated mouse serum and 5% unconjugated

mouse Fab' fragments (Pathak et al., 2007). Two-color confocal Z-series were acquired using sequential laser excitation under identical imaging conditions.

To analyze quantitatively the extent and intensities of glutamylated and acetylated tubulin in individual cilia, maximum intensity projection images were generated from deconvolved stacks using the Huygens Essential program (Scientific Volume Imaging, Hilversum, Netherlands) and saved in TIFF format. These maximum-intensity projections were viewed as composite color images using ImageJ (National Institutes of Health, Bethesda, MD), and lengths of lines drawn in separate red (glutamylated tubulin) and green (acetylated tubulin) channels were determined using the analyze function. The integrated pixel densities were similarly measured separately for red (glutamylated tubulin) and green (acetylated tubulin) channels in regions of interest drawn around individual cilia.

### High-speed videomicroscopy analysis

The 54-hpf control and morpholino-injected, 1-phenyl 2-thiourea-treated embryos of wild-type TuAB or CD-41 GFP transgenic zebrafish were maintained alive and anesthetized in E3 egg water containing tricaine (1:25 dilution of 4.1% stock). The immobilized larvae were placed on 3% methyl cellulose, immersed in the anesthetic mix, and observed by Nomarski optics using a 40×/0.55 water immersion lens mounted on the Nikon E-800 microscope. Images of moving cilia in distended pronephric lumens (due to cystic phenotype or mechanical obstruction induced in the distal pronephros of controls) were obtained using the Dragonfly2 CCD camera (Point Grey Research, Richmond, Canada) at speeds between 235 and 245 frames/s. To obtain kymograms from sequential movie frames, the entire stack of 324 × 242 images in QuickTime movies (Apple, Cupertino, CA) were imported in ImageJ software, and the line function was used to slice the movie frames along a small plane orthogonal to the axis (near the center of cilium) of cilia selected for analysis. Beat amplitude information was assessed from the kymograms by determining the length of pixels between the extreme positions of the cilium and converted to a micrometer scale (1 μm = 2.7 pixels in a 324 × 242 tiff image). The beat frequency was assessed by measuring the number of peaks in the kymogram corresponding to a 1-s-long movie.

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