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The Bardet–Biedl syndrome-related protein CCDC28B modulates mTORC2 function and interacts with SIN1 to control cilia length independently of the mTOR complex

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CCDC28B **encodes a coiled coil domain-containing protein involved in ciliogenesis that was originally identified as a second site modifier of the ciliopathy Bardet–Biedl syndrome. We have previously shown that the depletion of CCDC28B leads to shortened cilia; however, the mechanism underlying how this protein controls ciliary length is unknown. Here, we show that CCDC28B interacts with SIN1, a component of the mTOR complex 2 (mTORC2), and that this interaction is important both in the context of mTOR signaling and in a hitherto unknown, mTORC-independent role of SIN1 in cilia biology. We show that CCDC28B is a positive regulator of mTORC2, participating in its assembly/stability and modulating its activity, while not affecting mTORC1 function. Further, we show that Ccdc28b regulates cilia length *in vivo*, at least in part, through its interaction with Sin1. Importantly, depletion of Rictor, another core component of mTORC2, does not result in shortened cilia. Taken together, our findings implicate CCDC28B in the regulation of mTORC2, and uncover a novel function of SIN1 regulating cilia length that is likely independent of mTOR signaling.**

INTRODUCTION

Primary cilia are key organelles for mediating the interaction between cells and their environment, acting as transducers of a host of signals, including mechanical, chemical and paracrine signaling cascades. Not surprisingly, cilia dysfunction has been linked to the pathogenesis of a number of human genetic disorders, termed 'ciliopathies' (1, 2). Primary cilia are microtubule-based organelles that are formed and maintained through a transport mechanism, intraflagellar transport (IFT) (3). Ciliogenesis and cilia length are tightly regulated and although the precise mechanisms are not completely understood, several signaling cascades have been implicated to date including fibroblast growth factor, Notch and mTOR (mechanistic or mammalian target of rapamycin) (4–6).

The mTOR pathway is a central regulator of cellular homeostasis, modulating cell growth and proliferation (7). The mTOR Ser/Thr kinase can be found in two protein complexes, mTORC1 and mTORC2. mTORC1 promotes cell growth by regulating protein and lipid biosynthesis, while mTORC2 has a role in cytoskeletal organization and cell migration (7,8). The mTOR-cilia link is supported by several reports. First, depletion of the mTOR inhibitor *tsc1a* in zebrafish results in characteristic ciliary phenotypes such as the formation of kidney cysts and defects in left–right axis determination (9,10). Further, mTOR controls ciliary length through the regulation of protein synthesis (6). Finally, ciliary bending has been shown to be required to downregulate mTORC1 activity through the activation of the mTOR inhibitor LKB1 at the basal body (11).

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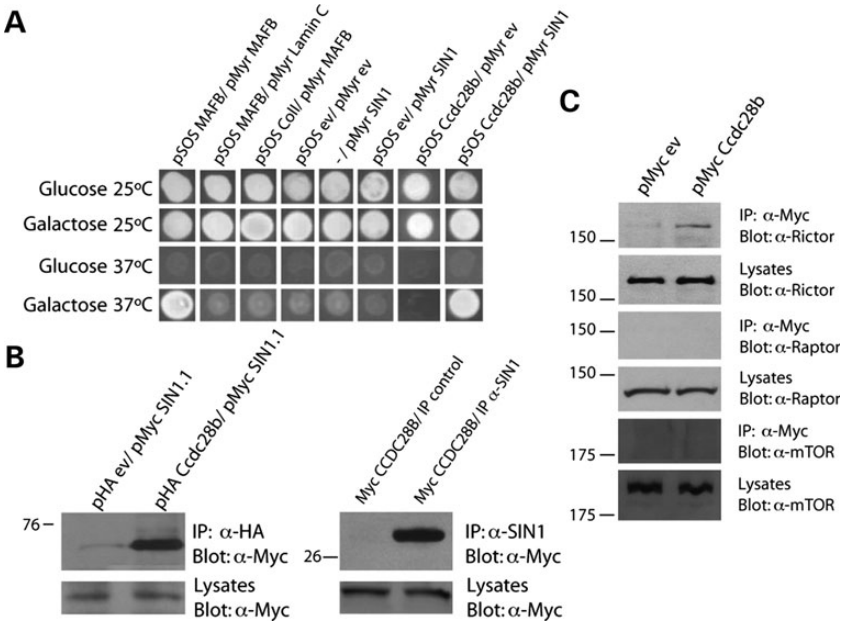

Figure 1. CCDC28B interacts with the mTORC2 components SIN1 and Rictor. (**A**) SIN1 confers yeast cells the ability to grow at the restrictive temperature of 37°C only in the presence of CCDC28B. MAFB–MAFB: positive control; MAFB–Lamin C, ColI-MAFB and pSOS ev–pMyr ev: negative controls. (**B**) Myc-SIN1.1 (long isoform) is detected after immunoprecipitating HA-CCDC28B (left). Myc-CCDC28B is also detected after immunoprecipitating with α-SIN1 antibody (right). Same cell lysates were used for IP controls and α-SIN1 thus controlling for initial levels of SIN1. (**C**) Rictor, but not Raptor or mTOR, is co-immunoprecipitated with Myc-CCDC28B. Same conditions were used in all cases. Molecular weights are indicated in kDa.

One ciliopathy is Bardet–Biedl syndrome (BBS, OMIM 209900), a disorder characterized by retinal degeneration, obesity, gonadal and renal malformations, mental retardation and polydactyly (12). BBS is caused by mutations in either of at least 17 genes (*BBS1-12*, *MKS1*, *CEP290, FRITZ/C2ORF86*, *SDCCAG8, LZTFL1*; Ref. 13 and references within). The BBS proteins participate in diverse aspects of ciliary biology, including cilia formation/maintenance and modulation of cilia-dependent signaling (14–20). In addition, a hypomorphic mutation in *CCDC28B* (coiled coil domain containing protein 28B), together with mutations at other BBS loci, was shown to correlate with a more severe presentation of the disease in some families (21). We have shown recently that knockdown of CCDC28B results in shortened cilia *in vitro* and *in vivo* (22). However, the mechanism by which CCDC28B participates in the regulation of cilia length and its overall biological function are unknown. Here, we show that CCDC28B interacts with SIN1, a structural member of the mTORC2 complex, and that this interaction modulates the assembly/stability and function of the complex both in cells and *in vivo* in zebrafish. We show that the ability of CCDC28B to regulate ciliary length depends on its interaction with SIN1 but, surprisingly, independently of mTORC2. Taken together, our data inform the cellular functions of the ciliopathy modifier CCDC28B and point to an mTORC-independent role for an mTORC2 core component in cilia length regulation.

RESULTS

CCDC28B interacts with the mTORC2 component SIN1

We performed a cytoplasmic yeast two-hybrid screen using CCDC28B as bait (pSOS-CCDC28B) and a fetal brain library as prey (pMyr construct) and identified the mTORC2 component SIN1 (also named MAPKAP1 for mitogen activated protein kinase associated protein 1) (23,24). Yeast cells carrying both CCDC28B and SIN1 were able to grow at the non-permissive temperature of 37°C (Fig. 1A). We confirmed this interaction in mammalian cells by co-immunoprecipitation (CoIP) using both epitope tagged proteins, HA-CCDC28B and Myc-SIN1.1 (SIN1 isoform 1; NM_001006617.1), and a semi-endogenous CoIP using a SIN1 antibody and Myc-CCDC28B (Fig. 1B). mTORC2 includes mTOR, mLST8 and Rictor and the stability of the complex depends on the SIN1–Rictor interaction (8,23,25). We therefore assessed whether CCDC28B can also interact with Rictor, mTOR or the mTORC1 specific component Raptor. We transfected cells with Myc-EV (empty vector) or Myc-CCDC28B, performed the CoIPs with a α-Myc antibody and tested for the presence of the different mTORC components by western blot. Using the same conditions in all cases, we were able to detect an interaction between CCDC28B and Rictor but not between CCDC28B and mTOR or the mTORC1 specific component Raptor (Fig. 1C). In addition, we immunoprecipitated Raptor and mTOR from cell lysates expressing Myc-CCDC28B. While we were able to co-immunoprecipitate mTOR with Raptor and Raptor with mTOR, again we did not obtain any evidence of Myc-CCDC28B being able to interact with either Raptor or mTOR (data no shown). Therefore, our results show that CCDC28B is able to interact with SIN1 and Rictor. Importantly, SIN1 and Rictor have been shown to form a heterodimer, which in turn interacts with mTOR to assemble mTORC2 (8,24,26). Thus, our results suggest that CCDC28B could participate in mTORC2 assembly.

CCDC28B is required for normal mTORC2 activity *in vitro* and *in vivo*

Next we evaluated if the activity of mTORC2 was affected by CCDC28B both in cultured cells and *in vivo* in *Danio rerio* (zebrafish). mTORC2 phosphorylates Akt in its hydrophobic domain at S473 (24,27), and thus we measured phospho-Akt S473 as a reporter of mTORC2 function. We transfected murine NIH3T3 cells with the short hairpin-expressing constructs pSUPER-Ccdc28b and pSUPER-ev (empty vector; Supplementary Material, Fig. S1), and measured the ratio of phospho-Akt S473 over total Akt. *Ccdc28b* knockdown cells showed a significant decrease in phospho-Akt S473 when compared with control cells (~40% reduction; $P < 0.01$; Fig. 2A). Comparable results were obtained using HeLa cells (Supplementary Material, Fig. S2). We then targeted the single zebrafish *CCDC28B* ortholog using our previously characterized splice blocking morpholino (MOspl) (22) and obtained total protein 48 hpf (hours post-fertilization). Depletion of Ccdc28b in zebrafish resulted in a marked reduction of phospho-Akt S473 (~80% reduction, $P < 0.01$; Fig. 2B). Consistent with these findings, phospho-Akt S473 was elevated significantly upon overexpression of Ccdc28b in both cultured cells and zebrafish: we observed a ~100% increase when we transfected cells with pMyc-Ccdc28b ($P < 0.01$; Fig. 2C) or injected *ccdc28b* mRNA into zebrafish embryos ($P < 0.01$; Fig. 2D). Although we did not find CCDC28B associated with Raptor, we nonetheless assessed mTORC1 activity (phosphorylation of 4E-BP1 at Thr37/46 and S6 at both Ser235/236 and Ser240/244) in cells and zebrafish: we did not observe significant differences (Fig. 2E and F). Taken together, our data show that CCDC28B is a positive regulator of mTORC2 but not mTORC1.

Depletion of CCDC28B results in disruption of mTORC2

To gain insight into the mechanism by which CCDC28B affects mTORC2, we tested total protein levels of Sin1 and Rictor in Ccdc28b knockdown cells: we did not observe significant changes (Supplementary Material, Fig. S3A). Next, we asked whether CCDC28B is required for mTORC2 complex assembly/stability. We transfected human Hek293 cells with control and previously tested *CCDC28B* double-stranded RNA oligos (22) and performed CoIPs assessing the interactions SIN1–mTOR and SIN1–Rictor (Fig. 3A and B). Knockdown of CCDC28B resulted in a reduction in the amount of Rictor and mTOR associated with SIN1 (Fig. 3D; $P < 0.01$). The overall levels of Rictor and mTOR were not affected (Supplementary Material, Fig. S3B). In contrast, the Raptor–mTOR interaction did not show a significant difference between targeted and control cells (Fig. 3C and D). Thus, knockdown of CCDC28B results in a reduction in mTORC2 levels, a finding that is consistent with the observed decrease in mTORC2 activity.

Sin1 affects Kupffer's vesicle cilia length independently of mTORC2

We have shown previously that Ccdc28b plays a role in ciliogenesis (22). In addition, the mTOR pathway has also been shown to participate in ciliogenesis and regulate cilia length (6,9,11). Thus, one possibility was that CCDC28B might regulate ciliary length through its interaction with SIN1 and the regulation of the mTORC2 pathway. However, while the recent reports linking mTOR signaling with ciliogenesis were centered on mTORC1, knockdown of CCDC28B affected only mTORC2. To discriminate between mTORC1 and mTORC2, we tested whether *raptor*, *sin1* or *rictor* can regulate cilia length individually. We designed splice-blocking morpholinos (MOspl) against *raptor*, *sin1* and *rictor* (see Supplementary Information). We injected different doses of each MO, tested their activity by RT-PCR (Supplementary Material, Fig. S4) and selected for further use those resulting in >80% of affected embryos with minimal lethality (<5%): 3 ng for *ccdc28b* (22), 1 ng for *raptor*, 3 ng for *sin1* and 4 ng for *rictor*. Importantly, in each MO injection, control embryos were injected with a standard MO (MOstd) at the highest dose used. To test the functionality of the MOs, we then assessed mTORC1 and mTORC2 activity in the morphants. As expected, knockdown of Raptor resulted in a significant reduction in mTORC1 function (49 ± 31% of control activity for P-S6(S235/236) and 23 ± 4.2% for P-S6(S240/244); $P < 0.01$; Fig. 4A), while knockdown of either Sin1 or Rictor affected the activity of mTORC2 (65 ± 11 and 27 ± 1% of control activity for P-Akt(S473), respectively; $P < 0.01$; Fig. 4A). Knockdown of *raptor* was reported to result in delayed gut development, whereas depletion of Rictor failed to affect this process and was not shown to produce other phenotypes (28). Likely highlighting differences in the dose of *raptor* morpholino tested (identical morpholinos were used), or in the design/efficiency of the *rictor* MOspl morpholino, we observed that *raptor* morphants were severely affected and characterized by a curved and shortened body axis, craniofacial defects and alterations in pigmentation (Fig. 4B). In contrast, Rictor knockdown, despite reduced mTORC2 activity, resulted in almost no gross phenotypes besides a mild reduction in head size, while Sin1 morphants presented a short body axis and reduced cranial length and width (Fig. 4B).

We studied cilia in the zebrafish Kupffer's vesicle (KV) using antibodies against γ- and α-acetylated tubulin. We measured at least 500 cilia from 18–20 KVs for each condition, comparing morphants with age-matched (10–12 somite stage) controls, and calculated average cilia length, standard error and *P*-value (*t*-test). Knockdown of Raptor resulted in a significant reduction in KV cilia length (4.16 ± 0.18 μm in controls to 2.46 ± 0.11 μm in the morphants; $P < 0.001$; Fig. 5A), consistent with previous reports (6). Importantly, the *sin1* MOspl also resulted in a significant reduction of cilia length (4.21 ± 0.16 μm in controls versus 3.11 ± 0.13 μm in morphants; $P < 0.001$; Fig. 5B). In contrast, the *rictor* morpholino did not affect the length of KV cilia (Fig. 5C). To confirm this, we designed translational blocking morpholinos (MOatg) for *rictor* and *sin1* thus targeting both zygotic and maternal mRNA. As expected, the overall phenotype was more severe: *sin1* morphants presented a shortened and curved body axis with clear craniofacial alterations, while rictor morphants were shorter than controls and presented marked craniofacial defects and hydrocephalus (Supplementary Material, Fig. S5A). Cilia in *sin1* MOatg morphants measured 3.41 ± 0.04 μm compared with 4.54 ± 0.08 μm in controls ($P < 0.001$), while *rictor* MOatg morphants presented cilia averaging 4.48 ± 0.07 μm compared with 4.27 ± 0.06 μm in controls (Supplementary Material, Fig. S5B). Therefore, knocking

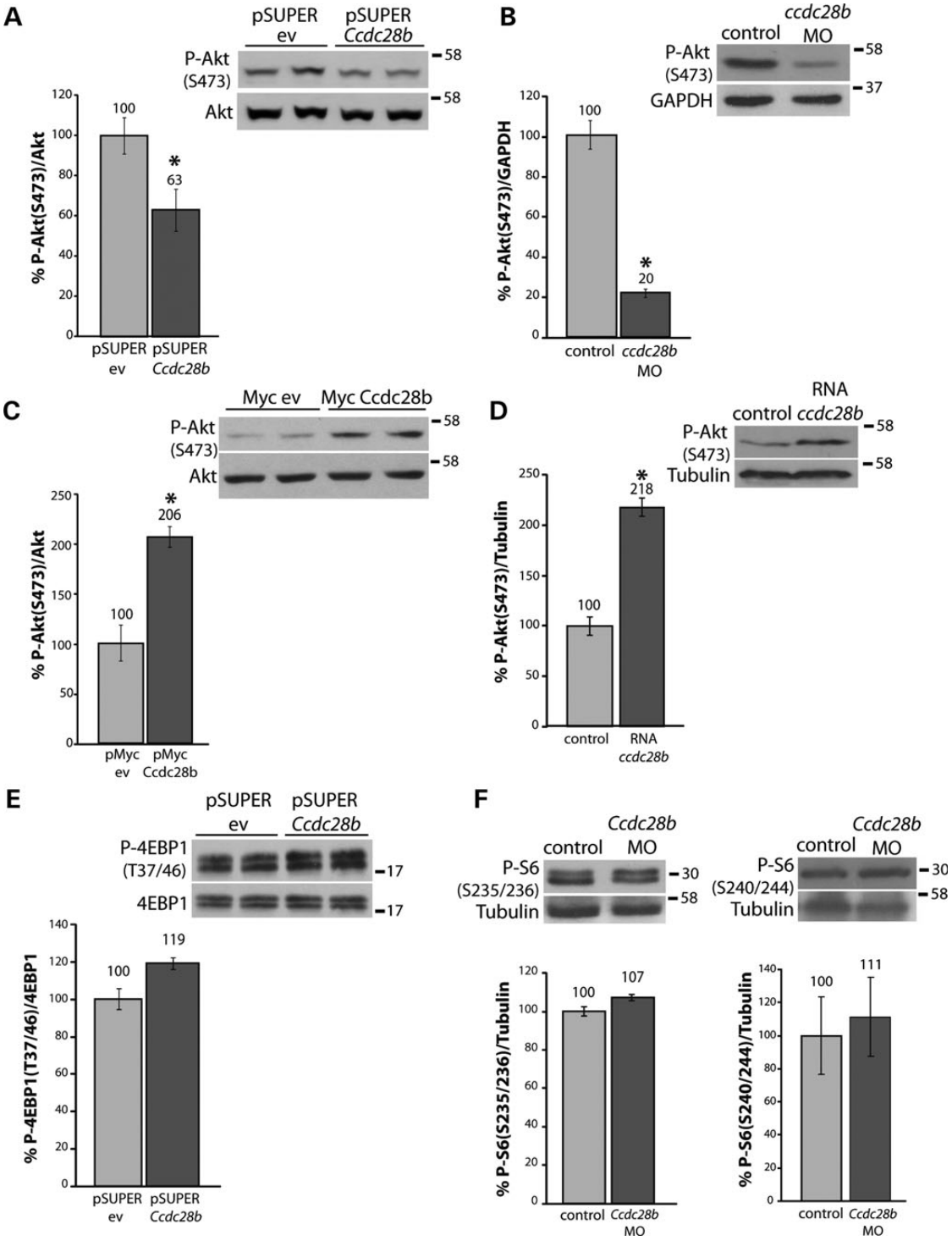

Figure 2. CCDC28B modulates mTORC2 activity. (**A** and **B**) Depletion of CCDC28B in both NIH3T3 cells (A) and zebrafish embryos (B) results in a reduction of phospho-Akt S473 indicating decreased mTORC2 activity. Total Akt or GAPDH were used as loading controls. (**C** and **D**) Overexpression of CCDC28B in cells (C) and in zebrafish (D) resulted in increased mTORC2 activity. Total Akt and tubulin were used as controls. (**E** and **F**) Depletion of Ccdc28b in cells (E) or *in vivo* (F) did not affect the activity of mTORC1, measuring P-4EBP1 (T37/46) or P-S6 (S235/236 and S240/244), respectively. Total 4EBP1 and Tubulin were used as loading controls. Asterisks indicate statistically significant differences ($P < 0.01$). Error bars correspond to SEM. Molecular weights are indicated in kDa.

down Rictor using MOspl or MOatg did not result in significant shortening of KV cilia, even at a dose of *rictor* MOspl that affected mTORC2 function to a greater extent than the *sin1* MOspl (Figs 4A and 5C). In addition, we transfected hTERT-RPE (retinal pigmented epithelial cells) with stealth double-stranded RNAi oligos targeting each of *SIN1*, *Rictor*, *Raptor*, *CCDC28B* and *IFT88* (Supplementary Material, Fig. S6 and Ref. 22) and assessed cilia length by measuring in

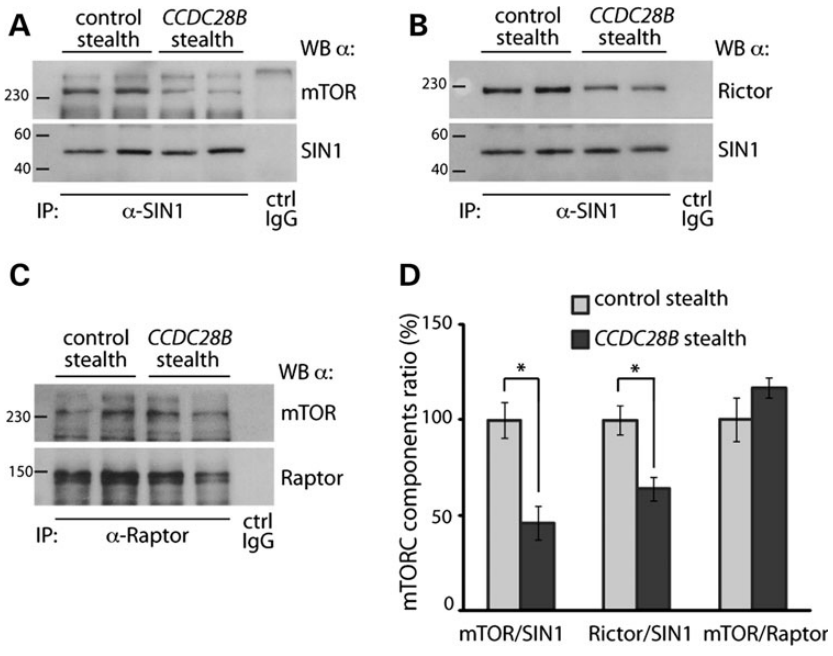

Figure 3. CCDC28B plays a role in mTORC2 assembly and/or stability. Immunoprecipitations using α-SIN1 from both control and CCDC28B knockdown cells (stealth) showed a significant reduction in the amounts of mTOR (**A**) and Rictor (**B**) in complex with SIN1. In contrast, immunoprecipitations with α-Raptor demonstrated that the interaction between Raptor–mTOR was not affected (**C**). The quantification of the western blot bands is shown (**D**). Asterisks mark significant differences ($P < 0.01$). Error bars correspond to SD. Molecular weights are indicated in kDa.

excess of 400 cilia per condition. Knockdown of Raptor and SIN1 resulted in a reduction in cilia length comparable with that obtained with the *IFT88* RNAi oligo: from 2.7 ± 0.05 μm in controls to 2.4 ± 0.06 μm in *Raptor*, 2.2 ± 0.05 μm in *SIN1* and 2.2 ± 0.04 in *IFT88* ($P < 0.001$; Fig. 5D). In contrast, the average cilia length in Rictor knockdown cells was not statistically different from controls at 2.6 ± 0.03 μm (Fig. 5D). Overall, our results show that the role of Sin1 in regulating cilia length is likely independent of mTORC2.

Ccdc28b interacts genetically with *sin1* to regulate ciliogenesis

Collectively, our results indicated that CCDC28B might regulate ciliary length through its interaction with SIN1 but independently from mTORC2. To test this, we performed a genetic interaction assay based on the hypothesis that partial disruption of a given pathway can generate a sensitized background on which mutations in more than one gene can synergize to produce a phenotype that cannot be explained by an additive effect. We thus used sub-optimal doses of *ccdc28b* and *sin1* MOspl to target each individual gene both separately and together and kept the total amount of MO constant across conditions with a standard control in the single injections. Injecting 2 ng of *ccdc28b* or 2 ng of *sin1* MOspl did not result in a significant ciliary defect (4.65 ± 0.21 μm controls, 4.61 ± 0.23 μm *ccdc28b* and 4.48 ± 0.21 μm *sin1*). In contrast, co-injecting these same doses of *ccdc28b* and *sin1* morpholinos resulted in a significant reduction of cilia length (3.26 ± 0.12 μm; $P < 0.001$; Fig. 6A). Thus, *ccdc28b* and *sin1* interact genetically to affect cilia length. We evaluated the levels of phospho-Akt S473 in these different injections. Importantly, changes in mTORC2 activity did not correlate with the observed cilia defects since injecting sub-optimal doses of *ccdc28b* and *sin1* MOspl, while did not affect cilia length, resulted in decreased mTORC2 activity (Fig. 6A).

We next tested for an interaction between *ccdc28b* and *rictor*. Injecting subotptimal doses of these morpholinos (2 and 3 ng respectively) alone or combined did not result in a significant shortening of cilia 5.0 ± 0.13 μm controls, 4.8 ± 0.09 μm *ccdc28b*, 5.2 ± 0.10 μm *rictor* and 5.1 ± 0.09 μm double (Supplementary Material, Fig. S7). We also tested for genetic interactions between *ccdc28b* and *raptor,* which by itself affects cilia length. Again, neither single or double injections of suboptimal doses of *ccdc28b* and *raptor* MOspl resulted in a significant shortening of KV cilia length: 4.82 ± 0.06 μm controls, 4.57 ± 0.07 μm *ccdc28b*, 4.51 ± 0.06 μm *raptor*, 4.57 ± 0.05 μm double (Fig. 6B). Thus, our data indicate that *ccdc28b* and *raptor* do not interact to regulate cilia length while support the specificity of the assay.

Ccdc28b regulates ciliogenesis upstream of SIN1

Finally, we performed reciprocal rescue experiments. We first compared cilia between embryos injected with standard MO (controls), *ccdc28b* mRNA, *sin1* MOspl and embryos that were co-injected with the *ccdc28b* mRNA and *sin1* MOspl. While we observed a significant shortening of cilia in the *sin1* morphants, overexpression of *ccdc28b* did not rescue the phenotype (Fig. 7A). Likewise, overexpression of *ccdc28b* did not rescue the cilia defect in *raptor* morphants (Supplementary Material, Fig. S8). We then performed the reciprocal experiment

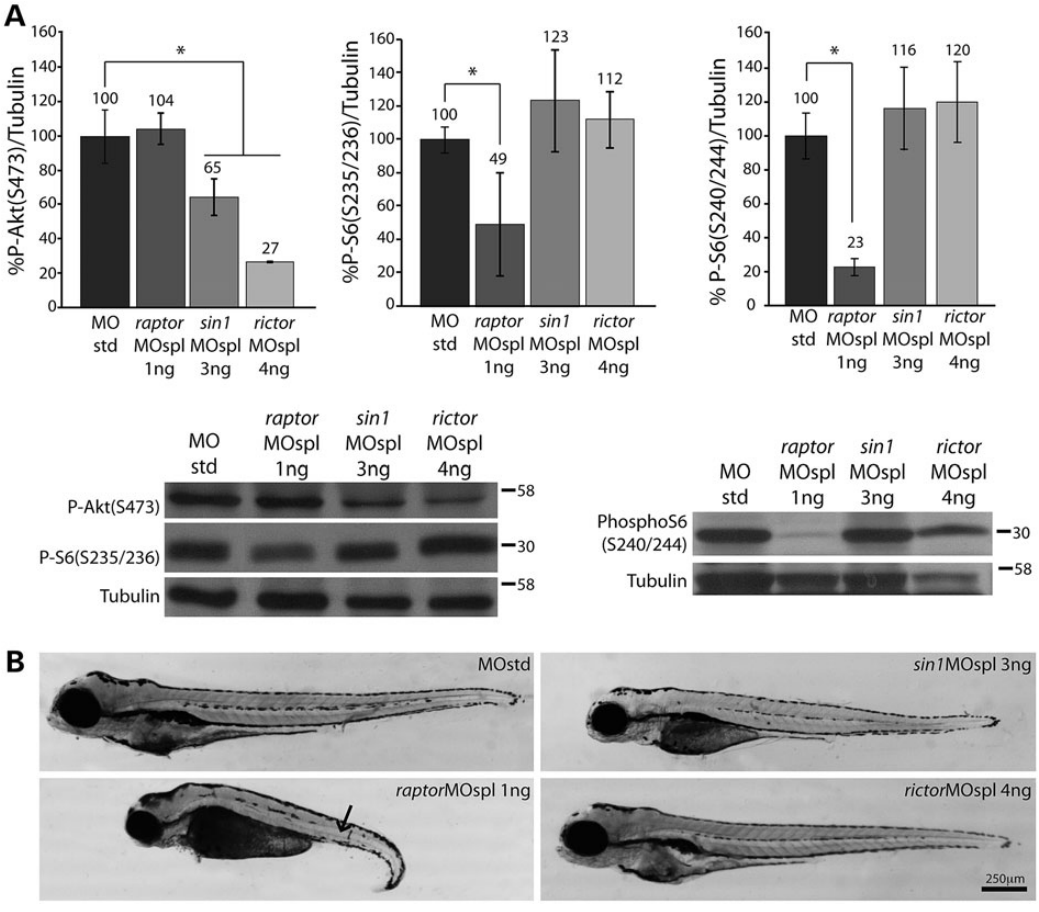

Figure 4. Knockdown of mTORC1 and mTORC2 components in zebrafish. (**A**) The activity of mTORC1 (P-S6 S235/236 and S240/244) and mTORC2 (P-Akt S473) was measured in each morphant. Knockdown of Raptor affected mTORC1 activity, while knockdown of Sin1 and Rictor resulted in reduced P-Akt S473. Tubulin was used as loading control. The results correspond to two independent injections. In each experiment, 25–30 embryos per condition were pooled to extract protein. 4 ng of MOstd were used in all cases. Asterisks denote statistically significant differences ($P < 0.01$). Error bars correspond to SEM. Representative westerns are shown and molecular weights are indicated in kDa. (**B**) Representative images of embryos injected with the control MOstd, *sin1*, *raptor* and *rictor* MOspl. *Raptor* morphants are characterized by a curved and shortened body axis, craniofacial defects and alterations in pigmentation (arrow). *Sin1* morphants are shorter than controls and present reduced cranial length and width. *Rictor* morphants have no obvious morphological phenotypes despite a marked reduction in mTORC2 activity. Embryos were assessed at 48 hpf. All images were taken using the same magnification.

overexpressing *sin1* in *ccdc28b* morphants. We observed a partial but significant rescue of cilia length from 3.75 ± 0.07 μm in *ccdc28b* morphants to 4.05 ± 0.05 μm upon co-injection with *sin1* mRNA ($P < 0.001$; Fig. 7B). Thus, our results indicate that Sin1 likely acts downstream of Ccdc28b.

DISCUSSION

The regulation of cilia length is critical for the function of the organelle and a number of proteins and signaling cascades have been implicated in this process (29). We have shown recently that CCDC28B is involved in regulating cilia length (22) and here we have continued with the functional characterization of this protein by attempting to identify proteins that interact with it in this process. We show that CCDC28B binds SIN1 directly and acts as a positive regulator of mTORC2 likely exerting this activity by affecting its assembly/stability. We also show that CCCD28B regulates cilia length, at least in part, through its interaction with SIN1 since *sin1* morphants present shortened KV cilia, *ccdc28b* and *sin1* interact genetically and Sin1 can partially rescue the cilia phenotype of *ccdc28b* morphants. Importantly, by targeting mTORC1 and mTORC2 independently, we show that while raptor/mTORC1 participates in regulating cilia length, the role of Sin1 in cilia appears to be independent of mTORC2: we did not observe defects in KV cilia in *rictor* morphants using either MOspl or MOatg, although the activity of mTORC2 was severely compromised in these embryos. Furthermore, knockdown of SIN1 and Raptor, but not Rictor, affected cilia length in a human-ciliated cell line. Our results further reinforce previous reports linking mTORC1 and ciliogenesis (6,9) and are consistent with a recent report showing that conditional knockout of *Rictor* in mouse kidney distal convoluted tubules does not result in cilia length defects (30). Therefore, we show for the first time that SIN1 is involved in the regulation of cilia length, a role that it achieves independently of mTORC2.

Besides its known role in the context of mTORC2, SIN1 was identified originally in *Schizosaccharomyces pombe* as an interactor of the Sty1 MAP kinase, a member of the yeast stress

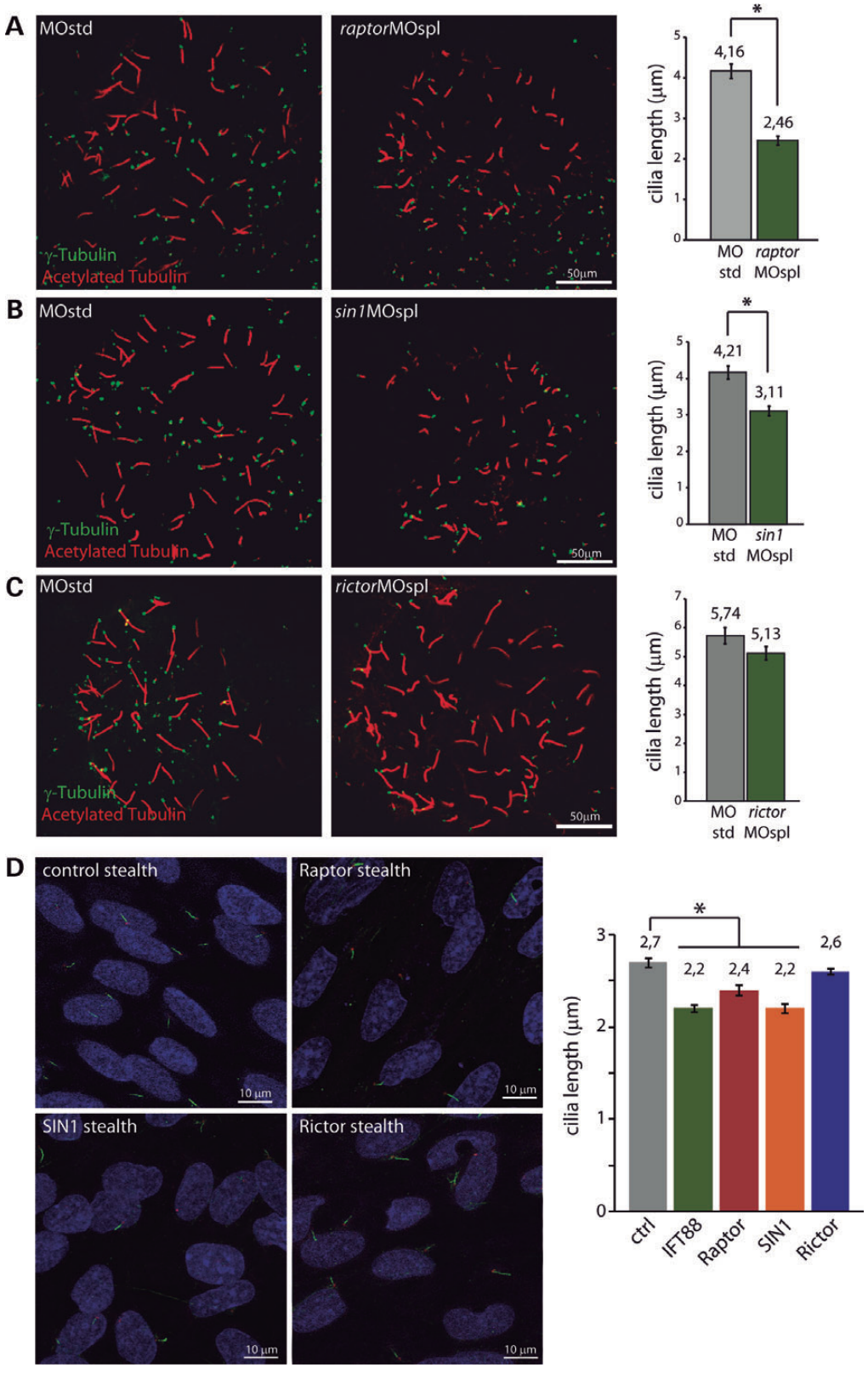

Figure 5. Sin1 affects cilia length independently of mTORC2. 18–20 KVs of each *raptor* (**A**) *sin1* (**B**) and *rictor* (**C**) splice morphants were analyzed by immunofluorescence and confocal microscopy using a γ-tubulin (green) and acetylated α-tubulin (red) antibodies to stain basal bodies and cilia, respectively. A standard MO (MOstd) was injected as control alongside each morpholino injection. In excess of 500 cilia were analyzed for each condition and the length of cilia was measured using ImageJ. The average cilia length is shown in the graphs. Both, knockdown of Raptor (A) and Sin1 (B), but not Rictor (C), resulted in a significant (asterisks) shortening of KV cilia. Error bars correspond to SEM; $P < 0.001$. (**D**) Cilia length was measured in hTERT-RPE cells transfected with stealth double-stranded RNAi oligos. Cilia and basal bodies were labeled using acetylated α-tubulin (green) and γ-tubulin (red), respectively. In excess of 400 cilia were analyzed per condition using ImageJ. Again, knockdown of Raptor and SIN1, but not Rictor, resulted in significantly (asterisk) shortened cilia. Knockdown of IFT88 was used as a control of the system. Error bars correspond to SEM; $P < 0.001$.

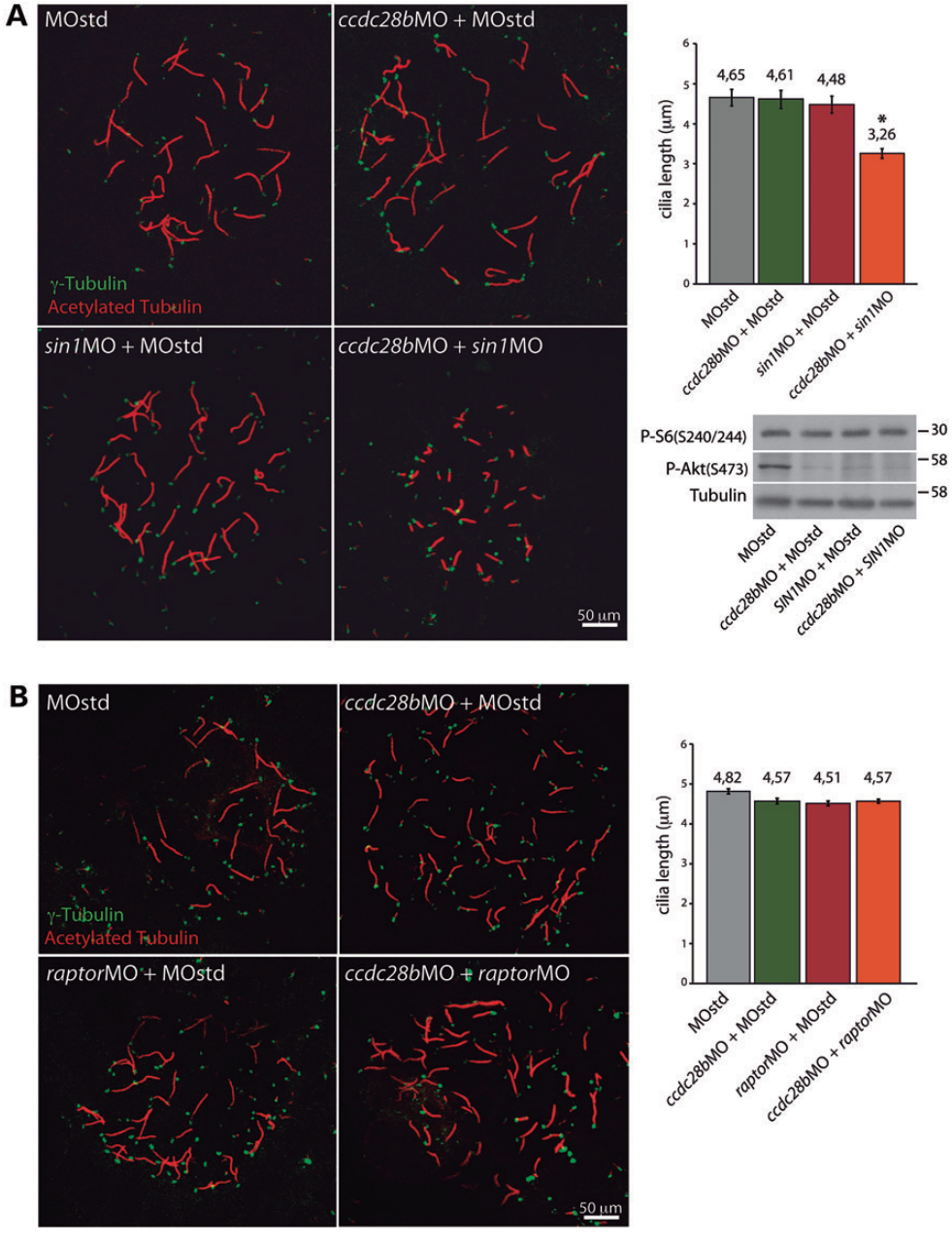

Figure 6. *ccdc28b* and *sin1* interact genetically. Sub-optimal doses of *ccdc28b*, *sin1* and *raptor* MOspl were used to test for genetic interactions between these genes. (**A**) Injection of either the control morpholino (MOstd), 2 ng of *ccdc28b* MOspl or 2 ng of *sin1* MOspl did not result in cilia shortening. However, co-injecting these suboptimal doses of *ccdc28b* and *sin1* MOspl resulted in significantly shorter KV cilia. The asterisk denotes statistically significant differences ($P < 0.001$). Importantly, this effect on cilia length is not explained by mTORC1 (P-S6 S240/244) or mTORC2 (P-Akt S473) dysfunction (western blots). Molecular weights are expressed in kDa. (**B**) In contrast, neither single nor double injections of the *ccdc28b* and *raptor* MOspl affected cilia length. Error bars correspond to SEM.

activated MAP kinase (SAPK) pathway (31). In mammalian cells, SIN1 interacts with and inhibits MEKK2, which activates different MAPKs (32,33). Moreover, SIN1 has also been shown to inhibit the activation of the ERK and JNK pathways through Ras (34). Therefore, SIN1 might regulate, at least indirectly, different MAPK signaling cascades. Importantly, the MAPKs have been implicated in the regulation of IFT and ciliogenesis generally associating impairment in MAPK signaling with increased cilia length (reviewed in 29). For example in *C. elegans*, the conserved MAPK DYF-5 regulates anterograde IFT movement and *dyf-5* loss of function results in elongated cilia (35). However, Abdul-Majeed *et al*. (36) have shown that inhibition of MEK1 results in shortened cilia in human cultured cells, suggesting that these kinases can act to increase or decrease cilia length depending on the cellular context, the specific MAPK and the organism. Therefore, CCDC28B might regulate ciliary length through its interaction with SIN1 and the consequent regulation of MAPK signaling.

CCDC28B might play a structural role for example mediating the interaction of SIN1 with other proteins. CCDC28B is a

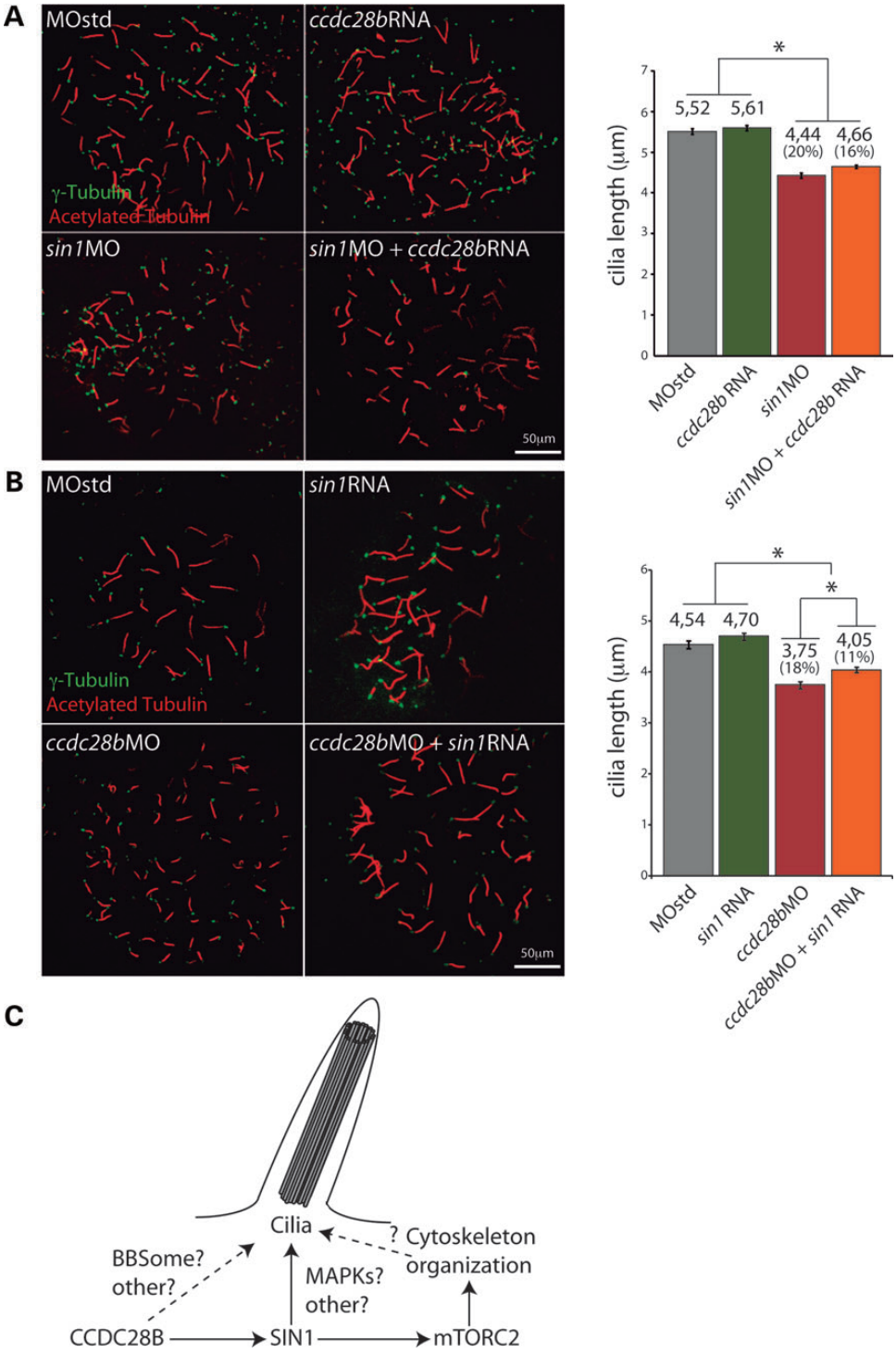

Figure 7. Ccdc28b affects ciliary length upstream of SIN1. Reciprocal rescue experiments to further characterize the *ccdc28b-sin1* interaction. (**A**) The KV cilia phenotype of *sin1* full dose morphants was not rescued by co-injection of *ccdc28b* mRNA. Also, overexpression of *ccdc28b* by mRNA injection did not affect cilia length by itself. (**B**) In contrast, while overexpression of *sin1* alone did not affect ciliary length, injecting *sin1* mRNA in *ccdc28b* morphants partially restored the length of KV cilia. The quantifications of average cilia length for each condition are shown. Asterisks denote statistically significant differences ($P < 0.001$). Error bars correspond to SEM. (**C**) Model summarizing the results. CCDC28B regulates cilia length at least in part through SIN1. In addition, CCDC28B modulates the activity of SIN1 in the context of mTORC2, acting as an agonist of the complex. Dashed lines indicate alternatives mentioned in the discussion.

200-amino acid protein predicted to have a C-terminal coiled coil domain, a motif typically involved in mediating protein–protein interactions (22,37). Overall, our data support the possibility of CCDC28B playing a role facilitating the activity of SIN1 both in the context of mTORC2, interacting with SIN1 and Rictor, as well as outside of it regulating cilia length

(Fig. 7C). Our rescue experiments support this scenario since we observed rescue of cilia length only when we overexpressed *sin1* mRNA in a *ccdc28b* morphant background, and not vice versa, indicating that Sin1 is likely to act downstream of Ccdc28b. However, cilia length restoration was only partial suggesting that Sin1 alone is not sufficient to restore function. The activity of Sin1 might depend on the presence of Ccdc28b, which thus becomes the limiting factor in the rescue experiment. In addition, this result might also indicate that Ccdc28b regulates cilia length at least in part independently of Sin1. CCDC28B interacts with different BBS proteins, some of which are part of a macromolecular complex termed the BBSome, which plays a role in ciliogenesis (15,17,21,38,39). Further work will be required to test whether the activity of the BBSome is affected in the absence of CCDC28B.

Lastly, our data suggest that while part of the phenotypes that characterize *ccdc28b* morphants are likely due to cilia defects, others might be caused by defective mTORC2 signaling, findings that are likely relevant to understand the modifier effect of CCDC28B in BBS. While mTORC2 is known to be involved in the reorganization of the actin cytoskeleton, and thus could affect the morphology of the cell and its polarization (8,25,40,41), the BBS proteins participate in the Wnt non-canonical planar cell polarity (PCP) pathway which is required to polarize and orient cells in the plane of a tissue (14,16,18,42). Thus, it is possible that impairment in mTORC2 signaling could exacerbate a PCP-dependent phenotype. We have reported that different ciliated tissues appear disorganized in *ccdc28b* morphants and also, that muscle F-actin fibers are affected upon knockdown of Ccdc28b (22), phenotypes that could be due to impaired mTORC2 signaling. *Ccdc28b* morphants also present hydrocephalus (22), a condition that can be caused by impaired cilia motility (43). Interestingly, *rictor* morphants, although having cilia of normal length at the KV, also present hydrocephalus. Thus, an intriguing possibility is that mTORC2 dysfunction might cause ciliary defects other than impaired ciliogenesis or shortened cilia: for example, it could affect cilia orientation, an important parameter for coordinated cilia-dependent movement of fluids in the brain ventricles (44). An in-depth characterization of the morphants presented in this work will be required to test this possibility and to evaluate other phenotypic consequences of mTORC2 dysfunction exploiting the advantages of the zebrafish model.

MATERIALS AND METHODS

Yeast two-hybrid screen

We performed the Cytotrap yeast two-hybrid screen following the manufacturer's instructions (Stratagene) using CCDC28B as bait (pSOS vector) and a human fetal brain library as prey (Stratagene).

Cell culture and transfections

We maintained Hek293, HeLa and NIH3T3 cells in Dulbecco's modified Eagle medium (DMEM; Invitrogen) and hTERT-RPE cells in a 1:1 mix of DMEM and F12 (Invitrogen) and 0.01 mg/ml hygromicin B, both supplemented with 10% fetal bovine serum, at 37°C in 5% CO_2 and performed transfections using either the Calcium Phosphate Transfection Kit (Invitrogen) for plasmids or Lipofectamine RNAiMax for stealth double-stranded RNAs (Invitrogen). hTERT-RPE cells for immunocytochemistry were grown on cover slips.

shRNA interference

We selected 19 nt target sequences in *Ccdc28b* using the siDESIGN center (Dharmacon) and cloned the hairpins into the pSUPER vector (OligoEngine). We transfected and harvested cells after 72 h for protein or RNA extraction. The stealth RNAi oligos were obtained from Invitrogen. The stealth RNAi oligos for *CCDC28B* and *IFT88* were validated previously (22) and by western blot for *SIN1*, *Rictor* and *Raptor*. For mock knockdowns, we use the Low GC Negative Control (Invitrogen). Oligos sequences are available upon request.

Co-immunoprecipitations

We transfected Hek 293 cells in 10 cm dishes and harvested them 48 h post-transfection to obtain cell lysates using CHAPS buffer (Hepes 40 mM pH7.4, NaCl 120 mM, EDTA 1 mM, NaF 50 mM, Na_3VO_4 0.5 mM, CHAPS 0.3%) supplemented with a protease inhibitor cocktail (Sigma). We incubated cell lysates with the appropriate antibody immobilized onto protein A/G sepharose beads (Invitrogen) overnight and under rotation at 4°C. For the mTORC components CoIPs, we transfected Hek293 cells, harvested 48 h later and incubated cell lysates with anti-SIN1 or anti-Raptor antibodies (Bethyl) for 90 min at 4°C, added protein A/G sepharose beads (Sigma) and incubated for another 90 min at 4°C under rotation.

Western blot and antibodies

We performed western blots with the corresponding primary antibodies and quantified the bands using the ImageJ software (Image Processing and Analysis in Java, NIH). Antibodies: SIN1, Rictor and Raptor from Bethyl Labs; mTOR, Akt, PhosphoAkt (S473), PhosphoS6 (S235-236), PhosphoS6 (S240/244), Phospho4E-BP1 (T37/46) and 4E-BP1 from Cell Signaling; GAPDH from Abcam; α-Tubulin from Sigma. For western blot analysis of zebrafish samples, we removed the yolk, froze 25–30 embryos in liquid nitrogen and homogenized samples using micropestles (Eppendorf).

Morpholinos

All morpholinos were obtained from Gene Tools. For sequences and design, see Supplementary Information. We injected wild-type zebrafish embryos at a one- to two-cell stage with 0,5-1 nanoliters of morpholino solutions prepared to deliver the concentrations stated in the text. For rescue experiments, we cloned the full-length zebrafish *ccdc28b* and *sin1* ORFs into pCS2 + (Invitrogen) and prepared mRNA using the Ambion mMessage mMachine SP6 kit.

Cilia analysis

For both KV cilia and hTERT-RPE cells, we stained basal bodies and cilia using anti-γ-tubulin and anti-acetylated α-tubulin

antibodies, respectively (Sigma), and isotype specific secondary antibodies (Invitrogen). Nuclei were stained with TOPRO (Invitrogen).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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