RanGTP regulates cilium formation and ciliary trafficking of a kinesin-II subunit independent of its nuclear functions

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Abstract

Kinesin is part of the microtubule (MT)-binding motor protein superfamily, which exerts crucial functions in cell division and intracellular transport in different organelles. The heterotrimeric kinesin-II, consisting of the kinesin like protein KIF3A/3B heterodimer and kinesin-associated protein KAP3, is highly conserved across species between the green alga *Chlamydomonas* and humans. It plays diverse roles in cargo transport including anterograde (base to tip) trafficking in cilium. However, the molecular determinants mediating trafficking of heterotrimeric kinesin-II itself is poorly understood. Using the unicellular eukaryote *Chlamydomonas* and mammalian cells, we show that RanGTP regulates ciliary trafficking of KAP3. We found the armadillo repeat region 6-9 (ARM6-9) of KAP3, required for its nuclear translocation, is sufficient for its targeting to the ciliary base. Given that KAP3 is essential for cilia formation and the emerging roles of RanGTP/importin β in ciliary protein targeting, we further investigate the effect of RanGTP in cilium length regulation in these two different systems. We demonstrate that precise control of RanGTP levels, revealed by different Ran mutants, is crucial for cilium formation and maintenance. Most importantly, we were able to segregate RanGTP regulation of ciliary protein incorporation from of its nuclear roles. Our work provides important support for the model that nuclear import mechanisms have been coopted for independent roles in ciliary import.

Key words: RanGTP, Kinesin-II, KAP3, nuclear import, ciliary targeting

Introduction

Cilia are microtubule-based protrusions with sensory and/or motile functions. In mammals, defects in assembly and maintenance of cilia results in a series of diseases called “ciliopathies” (Fliegauf et al., 2007). The assembly and maintenance of these organelles are dependent on anterograde and retrograde intraciliary transport (IFT) (Rosenbaum et al., 2002). Anterograde IFT, which moves from the base of a cilium to the tip, is driven by kinesin-II (kozminski et al., 1995; Cole et al., 1998), whereas retrograde IFT, which moves from the tip back to the base, is achieved by cytoplasmic dynein 1b (Signor et al., 1999; Pazour et al., 1999; Porter et al., 1999). The kinesin-II motor family is composed of a heterotrimeric KIF3A/KIF3A/KAP3 motor and a homodimeric KIF17 motor (Hirokawa et al. 2009). Unlike homodimeric KIF17, heterotrimeric kinesin-II is highly conserved, and loss of function in any component of heterotrimeric kinesin-II results in defective cilia in different organisms (Walther et al., 1994; Morris et al., 1997; Sarpal et al., 2004; Zhao et al., 2011). In addition to cilium formation, regulation and maintenance of cilium length is also dependent on the size and frequency of kinesin-II trains recruited to/entering cilia (Ludington et al., 2013; Engel et al., 2009). In addition to its central role in IFT and ciliogenesis, heterotrimeric kinesin-II has also been reported in other organelle transport events outside cilia. This includes anterograde transport from endoplasmic reticulum to the Golgi apparatus in *Xenopus* (Le Bot et al., 1998), retrograde transport from the Golgi to endoplasmic reticulum in HeLa cells (Stauber et al., 2006), and establishment of cell polarity during migration (Murawala et al., 2009). Furthermore, heterotrimeric kinesin-II is reported to play critical roles in mitosis (Fan et al., 2004; Haraguchi et al., 2006).

Intracellular localization of heterotrimeric Kinesin-II subunits is cell cycle dependent. Cilia assemble in quiescent cells and disassemble in dividing cells (Plotnikova et al., 2009). Heterotrimeric kinesin-II is localized in both cilia and basal body in ciliated cells. When cells enter mitosis and cilia retract, the non-motor subunit KAP is transported into nucleus before nuclear membrane break down in cells of sea urchin blastulae (Morris et al., 2004). During cytokinesis, the motor protein KIF3B is localized in the midbody (Fan et al., 2004). Macromolecules can’t freely go into or out of the cilium and nucleus because diffusion barriers exist at the ciliary base and the nuclear pore complex (Kee et al., 2012; Takao et al. 2014., Endicott et al., 2018). The fundamental question is how this conserved heterotrimeric kinesin-II complex traffics between different compartments (the cytoplasm, nucleus and cilium) and how these processes are regulated.

For nuclear transport from the cytoplasm, the nuclear pore complex (NPC) mediates active transport of proteins (Alber et al., 2007). Larger proteins (>50 kDa) generally require RanGTP and specific importin transport receptors to cross the NPC (Gorlich et al., 1994; Gorlich and vogel et al., 1995). Importins usually bind a nuclear localization signal (NLS)-containing cargo at relatively low RanGTP level in cytosol. This moves the complex through NPCs and releases cargo in the nucleus where RanGTP concentration is high (Moore et al.,1993; Gorlich et al., 1999). In most cases, importin β1 binds to importin α, which interacts with a conventional NLS, to mediate substrate nuclear import (Gorlich and Kostka et al., 1995). In contrast, importin β2 recognizes non-traditional proline-tyrosine NLS (PY-NLS) for nuclear import (Lee et al., 2006). In the nucleus, direct binding of RanGTP with importin β results in cargo release (Gorlich et al., 1997).

Compared to nucleo-cytoplasmic transport, the molecular mechanisms controlling protein trafficking into cilia are less well understood. Several ciliary localization sequences were reported in previous studies including RVxP, VxPx, and Ax[S/A]xQ motifs that are important for mediating ciliary trafficking of membrane proteins (Jenkins et al., 2006; Geng et al., 2006; Mazelova et al., 2009; Berbari et al., 2008). It was proposed that there are shared mechanisms between ciliary import and nuclear import (Dishinger et al., 2010; Takao et al., 2014; Del Viso et al., 2016; Takao et al., 2017; Endicott et al., 2018). Several results indicated that RanGTP/importinβ/NLS import system is required for ciliary targeting of either membrane or soluble proteins including importin β1 for Crumsb3 (Fan et al., 2007), RanGTP/importin β2 for KIF17 (Dishinger et al., 2010), importin β2 for RP2 (Hurd et al., 2011), importin α1/α6/NLS for KIF17 (Funabashi et al., 2017), and importin β2/PY-NLS for GLI2/GLI3 (Han et al., 2017). It was also reported that importin β2/Rab8 forms a ternary complex with ciliary localization sequences to direct membrane protein trafficking to cilia (Madugula et al., 2016), suggesting that this process is independent of RanGTP and a NLS or PY-NLS. Despite these advancements in uncovering mechanisms for ciliary import, it remains unclear whether RanGTP regulates ciliary trafficking directly or by affecting nuclear import to result in defective ciliary trafficking. It is also unclear whether different cargoes depend on different importin β receptors for ciliary trafficking. Lastly, prior work investigated determinants of ciliary entry for the homodimeric ciliary kinesin composed of KIF17 (Dishinger et al., 2010), the heterotrimeric kinesin-II is the motor that dictates cilium assembly and maintenance (Walther et al., 1994; Morris et al., 1997; Sarpal et al., 2004; Zhao et al., 2011; Engel et al., 2009; Ludington et al., 2013). Therefore, we focused on how ciliary trafficking of the heterotrimeric kinesin-II is regulated by leveraging the unique

advantages of the unicellular green alga *Chlamydomonas reinhardtii* as an excellent eukaryotic model to study ciliogenesis (Harris et al., 2001; Rosenbaum et al., 2002).

Cilia of *Chlamydomonas* can be regenerated to full length in two hours, and unlike mammalian cells, ciliary assembly does not need to be induced (Rosenbaum et al., 1969) to result in heterogeneous population of ciliated and non-ciliated cells. The molecular mechanism of the nucleo-cytoplasmic trafficking is likely conserved between the *Chlamydomonas* and humans (Li et al., 2018). However, there are fewer constituent nucleoporins in the *Chlamydomonas* nuclear pore complex compared to that of humans (Neumann et al., 2006). By using both mammalian and *Chlamydomonas* cells, we have found that ciliary trafficking of kinesin associated protein KAP3 is regulated by RanGTP. We demonstrated that precise manipulation of RanGTP level is crucial for regulating cilium formation. Importantly, we were able to clearly show that RanGTP plays a direct role in incorporation of ciliary proteins that is independent of its nuclear roles. These results provide potential insights for the molecular mechanism orchestrating multi-compartment trafficking of the heterotrimeric kinesin-II motor complex. Further, they answer a long-standing open question in the field about whether nuclear import mechanisms have been coopted for *direct* ciliary import.

Results

Ciliary protein KAP3 can localize to the nucleus

The heterotrimeric kinesin-II motor complex consists of the heterodimeric motor proteins KIF3A/3B and the adaptor protein KAP3. In contrast, the homodimeric kinesin-II KIF17 motor does not need an adaptor protein to exert its function. Although it was suggested that KAP3 functions as a linker between KIF3A/3B and the specific cargoes to facilitate intracellular transport, the function of KAP3 is still not well characterized. First, we investigated the intracellular localization of KAP3 in ciliated and non-ciliated cells. HA tagged KAP3A and KAP3B (a short isoform of KAP3A) were transfected into RPE cells. After 24 hours, cilia were induced by serum starvation. As expected, KAP3 co-localized with acetylated-α-tubulin, confirming KAP3 is not affected by small HA epitope and can be targeted to cilia (**Figure 1A**). We also noticed that a significant amount of KAP3 was distributed throughout the nucleus (**Figure 1A**). We further examined the localization of KAP3A and KAP3B in different cell types. As shown in **Figure 1B,** KAP3A and KAP3B are mainly localized in the nucleus of COS-7 cells, although a small amount are distributed in the cytoplasm. We also showed that EGFP-tagged KAP3A and KAP3B could localize to the nucleus of MDCK cells **(Figure 1C)**. Nuclear

localization of KAP3A was consistent with that of EGFP tagged KAP3A in a previous report (Tenny et al., 2016).

To dissect critical regions within KAP3A responsible for its nuclear localization, as depicted in **Figure 1D**, a series of EGFP-fused KAP3 truncations was constructed (henceforth, KAP3 refers to the long isoform KAP3A). MDCK cells were transfected with these constructs, and subcellular distributions of KAP3 truncations were analyzed via fluorescence microscopy. As shown in **Figure 1E**, the N-terminal fragment KAP3 (1-270) and the C-terminal fragment KAP3 (661-792) are distributed in both the cytoplasm and nucleus which are similar to the EGFP control, and KAP3 (271-460) was exclusively distributed in the cytoplasm. In contrast, KAP3 (461-660), consisting of armadillo repeats (ARM) 6-9, were predominantly localized in the nucleus, which is similar to full-length KAP3. These data indicate that the region between amino acids 461 and 660 is crucial for nuclear localization of KAP3. Taken together, our data demonstrate that ciliary protein KAP3 can localize to the nucleus under the control of armadillo repeats 6-9.

RanGTP, but not importin β2, mediates nuclear translocation of KAP3

As shown in **Figure 1**, KAP3 is distributed to the nucleus in different cells. To determine the molecular mechanism of KAP3 nuclear translocation, we tested a well-studied pathway for protein nuclear import, RanGTP mediated nuclear import, which requires a high concentration of RanGTP in the nucleus for the disassembly of the imported complexes. To determine whether RanGTP drives nuclear import of KAP3, the dominant negative mutant RanQ69L which cannot hydrolyze GTP, was used in this study. As shown in **Figure 2A**, Ectopic expression of mCherry tagged RanQ69L blocked nuclear localization of EGFP tagged KAP3 in COS-7 cells, resulting in cytoplasmic distribution of KAP3. This data suggests that nuclear translocation of KAP3 is mediated by a RanGTP-dependent nuclear import pathway.

We mapped the region responsible for nuclear localization of KAP3 and found that KAP3 (471-660) is required. If the region we mapped is correct, the nuclear localization of this truncation KAP3 (471-660) should be RanGTP-dependent, which is the same as full-length KAP3. To test this, we co-transfected KAP3 (471-660) with the dominant negative Ran mutant RanQ69L. As shown in **Figure 2B**, RanQ69L completely disrupted nuclear import of KAP3 (471-660) and resulted in the cytoplasmic localization of this truncation. These results suggest that RanGTP-dependent nuclear import of KAP3 is dependent on the 471-660 region of KAP3.

We further examined which transport receptor is utilized for nuclear translocation of KAP3. Compared to the control, the importin β2 inhibitory peptide M9M did not block the nuclear

localization of KAP3 **(Figure 2C).** This data suggests nuclear localization of KAP3 is importin β2-independent.

The armadillo repeat domain (ARM6-9) alone is sufficient for ciliary base localization.

KAP3 can localize in both the cilium and nucleus. We dissected the regions required for nuclear translocation of KAP3. Next, we mapped the regions required for ciliary targeting of KAP3 in hTERT-RPE cells. As depicted in **Figure 3A**, full-length KAP3A contains three regions: a non-conserved N-terminal domain, nine armadillo repeats, and a C-terminal conserved domain (Jimbo et al., 2002; Shimuzu et al., 1996). The armadillo repeats are responsible for binding to KIF3A/3B, and the C-terminal conserved domain is responsible for specific cargo binding **(**Haraguci et al., 2005; Nagata et al., 1998; Jimbo et al., 2002; Deacon et al, 2003**)**. Based on this, a series of truncations of KAP3A were generated and intracellular localization of these truncations was examined after cilium induction. As shown in **Figure 3B**, the truncation KAP3A (661-792) completely abolished localization to the cilium and ciliary base. In contrast, the KAP3A (186-660) truncation showed intense signal at the ciliary base. The armadillo repeats are required for interaction with the motor subunit KIF3A and KIF3B, which suggests that the heterodimeric KIF3A/3B may play a role in KAP3 targeting to the ciliary base. It is noteworthy that cells expressing the truncated KAP3A (186-660), containing only the armadillo repeat domain, have normal cilia length, whereas cells expressing the truncated KAP3 (186-792), containing both the armadillo repeats and cargo-binding domains, have no cilia. Given that loss of the cargo-binding domain dramatically decreases KAP3 binding to KIF3A/3B (Haraguci et al., 2005), these data suggest that a dominant negative function of KAP3 truncations is dependent on their binding ability to the KIF3A/KIF3B motor subunits. Further mapping demonstrated that the armadillo repeat domain (ARM6-9), required for nuclear trafficking of KAP3A, is sufficient for ciliary base targeting (**Figure 3B**). Taken together, the armadillo repeats (ARM6-9) alone enable targeting of KAP3 to the ciliary base, probably through interacting with heterodimeric motor KIF3A/KIF3B and RanGTP.

RanGTP regulates cilium assembly in human retinal epithelial cells

KAP3 is a crucial regulator for anterograde IFT and cilium formation (Sarpal et al., 2003; Mueller et al., 2004). Our data revealed that RanGTP regulates nuclear translocation of KAP3 via its middle region, 471-660, which is sufficient for its targeting to the cilium base. Given the reported shared mechanisms between nuclear and ciliary import processes (Dishinger et al., 2010; Takao et al., 2014; Del Viso et al., 2016; Takao et al., 2017; Endicott et al., 2018) and

conflicting conclusions about the effect of RanGTP on cilium length regulation (Dishinger et al., 2010; Fan et al., 2011; Torrado et al., 2016), we were interested in determining whether RanGTP can affect cilium length in hTERT-RPE cells. Wild-type Ran and three well-characterized dominant negative Ran mutants (RanQ69L, RanG19V and RanT24N) were used in this study. First, we studied the intracellular localization of these proteins in hTERT-RPE cells. As shown in **Figure 4A**, all the mutants are predominantly localized in the nucleus which is similar to that of wild-type Ran. This data indicates that expression of these point mutants did not affect intracellular localization of Ran. To analyze the role of these mutants in cilium length regulation in hTERT-RPE cells, wild-type and mutant Ran expression plasmids were transfected into hTERT-RPE cells. 24 hours post-transfection, serum was depleted for another 24 hours for cilia biogenesis and measurement. As shown in **Figure 4B**, ectopic expression of wild-type Ran or the GDP-locked Ran mutant Ran T24N had no obvious effect on cilia length regulation. Cells transfected with dominant negative form of GTP bound Ran with relative low affinity to GTP (Lounsburg et al., 1996), RanG19V, showed normal cilia length but reduced ciliation percentage (**Figure 4C**) compared the un-transfected control cells. RanQ69L has higher affinity to GTP and resulted in a reduction in percentage ciliation in hTERT-RPE cells compared to RanG19V and RanT24N (**Figure 4C**). These data indicate that the RanGTP level in hTERT-RPE cells is a determinant of cilium formation. Taken together, the ability to bind and hydrolyze GTP by Ran in vivo, revealed by different dominant Ran mutants, regulates its essential functions on cilium assembly.

To determine why there is reduced cilium formation in RanQ69L-expressing RPE cells, we further investigated the localization of other important components, like the IFT complex and kinesin-II. As shown in **Figure 4D,** IFT81, a component of the IFT-B complex, is still localized in the ciliary base of RPE cells expressing RanQ69L, suggesting that IFT-B targeting is not affected and is unlikely to be the primary cause of defective cilium formation. In addition, RanQ69L expression didn't cause mislocalization of Cep164, the essential mother centriole distal appendage protein that could trigger ciliogenesis (Čajánek et al., 2014). To our surprise, the kinesin-II associated protein KAP3 didn't localize to the ciliary base. This data suggests that, in addition to potential roles for RanGTP in ciliary entry, ciliary targeting of the heterotrimeric kinesin-II is also RanGTP-dependent.

RanGTP regulates ciliary length and ciliary trafficking of KAP in *Chlamydomonas*

To see if mechanisms of Ran-dependent ciliary targeting and entry are broadly conserved, we tested the effect of Ran manipulation on assembly and kinesin motor targeting in

Chlamydomonas cilia. The unicellular green algae *Chlamydomonas* is an excellent model to study ciliary length regulation and protein trafficking. In addition to the extensive body of literature on motor trafficking and ciliary assembly in this organism, the small G-protein Ran is conserved between humans and *Chlamydomonas.* We therefore examined the role of Ran-like protein 1 (Ran1), the ortholog of human Ran, on ciliary length regulation in wild-type *Chlamydomonas* CC-125 cells. Disruption of Ran1-importin β interaction by small molecular inhibitor IPZ for 2 hours shortens ciliary length in a dose-dependent manner (**Figures 5A, B**).

To exclude that the phenotype was caused by the off-target effect of the inhibitor, the GTP-locked Ran1 mutant Ran1Q73L, corresponding to human RanQ69L, was transformed into *Chlamydomonas.* As shown in **Figure 5C**, there is strong Ran1Q73L expression as expected in *Chlamydomonas* (green arrow), despite a portion of expressed Ran1Q73L-zeocin fusion proteins being incompletely cleaved due to the efficiency of 2A peptide (red arrow). Compared to control cells with normal ciliary length and cell division, the cells expressing high levels of Ran1Q73L exhibits either clumpy cells or shortened ciliary length. One possible explanation for clumpy cells may be that there are no cilia to secrete ectosomes containing lytic enzyme to break the cell wall after cell division (Wood et al., 2013). These results demonstrated that RanGTP also plays pivotal roles in ciliary length regulation in *Chlamydomonas.*

We showed that KAP3, but not IFT81 and Cep164, couldn't be targeted to ciliary base in RPE cells constitutively expressing GTP-locked Ran. It was also reported that RanGTP regulates ciliary entry of the other motor KIF17, which is localized in the nucleus as KAP3 (Dishinger et al., 2010). Based on these data, we tested whether perturbing Ran function affected ciliary targeting or entry of KAP, the ortholog of human KAP3, in the *Chlamydomonas* KAP-GFP reporter strain CC-4296. As shown in **Figure 5D**, in control cells treated with DMSO, KAP-GFP is distributed in both the cilium (pink arrows) and cilium base. In contrast, in cells treated with IPZ, KAP-GFP ciliary entry is impaired (yellow arrows)**.** These results indicate that ciliary entry of KAP is regulated by RanGTP and importin β in *Chlamydomonas*.

RanGTP directly regulates ciliary protein incorporation during cilium regeneration in *Chlamydomonas*

One advantage of the *Chlamydomonas* model system in this context is the significant available information about requirements for nuclear regulation of ciliary assembly. During ciliary regeneration after ciliary severing (deciliation), new ciliary proteins need to be synthesized and transported to ciliary assembly sites for incorporation into cilia. This process requires initiating gene expression, which would be dependent on nuclear import. Therefore, it is possible that

RanGTP regulates cilium length by indirectly affecting nuclear import and ultimately new transcription/ciliary protein synthesis. To tease apart nuclear and non-nuclear effects, we were able to use the small molecular inhibitor cycloheximide (CHX) to inhibit new protein synthesis during cilium regeneration. In wild-type *Chlamydomonas* cells, this typically results in growth of cilia to 6 μm which exhibits the ability of these cells to incorporate already-synthesized proteins to generate half-length cilia without requiring transcription of new ciliary genes (Rosenbaum et al., 1969). As expected, when blocking new protein synthesis with CHX, the existing ciliary proteins can build a short cilium as shown in **Figure 6 A** and **A'**. However, when the deciliated cells are treated with CHX and IPZ to inhibit Ran function, there is no cilium formation during regeneration. This demonstrates that IPZ can directly impact incorporation of the "precursor pool" of proteins into cilia for assembly. To confirm that the lack of ciliary growth wasn't due to cell toxicity and that IPZ only impacts the ability of existing proteins to enter cilia, we washed out IPZ but still continued CHX treatment to inhibit new protein synthesis. As shown in **Figure 6 B and B'**, ciliary biogenesis is restored upon IPZ washout. These data clearly showed that RanGTP has direct effects in regulating cilium protein incorporation. During regeneration, IPZ also can block cilia formation in the presence of newly synthesized proteins (Figure 6C and C'). Given the dual role of RanGTP in mediating ciliary protein import and nuclear import, it is important to segregate nuclear and direct ciliary effects of Ran perturbation. Here we are able to show that in spite of its demonstrated roles in regulating nuclear protein import, RanGTP has direct roles in mediating ciliary protein incorporation for cilium formation.

Discussion

Although most kinesin motors are localized in the cytoplasm, different conditions allow some kinesin motors to transport into the nucleus including KAP3, KIF4, KIF17, and KIF17B (Morris et al., 2004; Seungoh et al., 2001; Dishinger et al., 2010; Macho et al., 2002). KAP3 and KIF4 can redistribute to the nucleus during mitosis (Morris et al., 2004; Seungoh et al., 2001). During mouse spermatid development, KIF17B shuttles from nucleus to cytoplasm (Macho et al., 2002). We observed that both the isoforms of the heterotrimeric kinesin-II accessory subunit KAP3A and KAP3B are localized in the nucleus, and that their nuclear localization is RanGTP dependent. Considering KIF17B can function as a transcription regulator (Macho et al., 2002), it is possible that KAP3 participates in regulation of gene expression in the nucleus. However, nuclear roles of KAP3 need to be further investigated.

Several lines of evidence suggest that RanGTP is involved in ciliary protein trafficking (Dishinger et al., 2010; Hurd et al., 2001; Fan et al., 2011; Maiuri et al., 2013). RanGTP was

reported to regulate ciliary entry of the homodimeric motor KIF17 and RP2 (Dishinger et al., 2010; Hurd et al., 2001). RanGTP was also reported to facilitate ciliary export of huntingtin Maiuri et al., 2013). However, there are several inconsistent conclusions about the role of RanGTP on cilium length regulation. Two groups demonstrated that RanGTP has no effect on ciliary length regulation (Dishinger et al., 2010; Torrado et al., 2016), whereas another group showed that manipulation of RanGTP concentration via RanBP1 knockdown could drive cilia formation in MDCK cells (Fan et al., 2011). Our results indicate that different dominant negative forms of Ran have different effects on cilia length regulation. The GTP-locked mutant RanG19V and GDP-locked mutant RanT24N have no effect on regulating cilium length. In contrast, another GTP locked mutant RanQ69L more dramatically affects cilium formation. The difference between RanQ69L and RanG19V is that RanQ69L has much higher affinity for GTP than RanG19V, thus RanQ69L-expressing cells have less free RanGTP than RanG19V-expressing cells. Taken together, our results suggest that precise manipulation of intracellular free RanGTP is critical for regulating cilium formation.

In addition to RanGTP, the importin transport receptors also participate in ciliary protein trafficking (Fan et al., 2007; Dishinger et al., 2010; Hurd et al., 2001; Torrado et al., 2016; Madugula et al., 2016; Han et al., 2017). There is also some disagreement about which importin is utilized for ciliary protein trafficking. Importin β1 was responsible for transmembrane protein Crumbs3 ciliary trafficking (Fan et al., 2007), and importin β2 was identified as the transport receptor for ciliary targeting of either transmembrane or soluble proteins like KIF17, Gli2 and GLi3. (Dishinger et al., 2010; Hurd et al., 2001; Madugula et al., 2016; Torrado et al., 2016; Han et al., 2017). However, additional data has shown that importin α1 and α6, but not importin β2, are responsible for ciliary targeting of soluble KIF17 (Funabashi et al., 2017). In general, importin β1, alone or in cooperation with importin α, transports substrates with a conventional NLS (Lange et al., 2007), whereas importin β2 transports substrates that contain the non-conventional PY-NLS (Lee et al., 2006). Consistent with this, ciliary targeting of the transcriptional factor Gli2/Gli3, which utilizes transport receptor importin β2, relies on its PY-NLS motif. PY-NLS mutations also result in the loss of Gli2/Gli3 ciliary targeting (Han et al., 2017). It is reported that the NLS-like sequence in the C-terminal region of KIF17 is required for its ciliary targeting (Dishinger et al., 2010). This NLS-like sequence was further confirmed as a classical mono-partite NLS (Funabashi et al., 2017). However, we noticed that PL, the PY variant, is located in the immediate downstream region of this NLS. So it is worth investigating whether or not this C-terminal NLS of KIF17 is a PY-NLS and which importin is used for KIF17 ciliary trafficking. Recently, a ternary complex consisting of importin β2, small GTPase Rab8 and

ciliary targeting signals was reported to guide transmembrane protein trafficking to cilium (Madugula et al., 2016). This data suggests that spatial structure of the ternary complex, but not specific ciliary targeting sequences, are required for ciliary targeting of membrane proteins. This highlights that the detailed working model for how importin mediates ciliary import needs to be further clarified.

There is increasing data that nuclear import and ciliary import shares similar mechanisms, at least in part (Dishinger et al., 2010; Kee et al., 2012; Takao et al., 2014; Del Viso et al., 2016; Takao et al., 2017; Endicott et al., 2018). First, both the nuclear pore complex and the ciliary pore complex form a diffusion barrier (Kee et al., 2012; Endicott et al., 2018). Second, the RanGTP/importin transport system is also used for ciliary protein trafficking (Fan et al., 2007; Dishinger et al., 2010; Hurd et al., 2011; Han et al., 2017). Third, some nucleoporins also localize in the ciliary base to regulate barrier diffusion ability (Dishinger et al., 2010; Del Viso et al., 2016; Endicott et al., 2018). Our data have shown that RanGTP can regulate cilium formation and ciliary trafficking of KAP3. One remaining critical question is whether RanGTP has direct effects in modulating ciliary protein transport. One possibility is that the effect of RanGTP is an indirect result of inhibiting nuclear import of proteins, like transcriptional factors, which are required for ciliary length regulation. By using the unicellular green alga *Chlamydomonas* as a model organism, we clearly demonstrated that RanGTP function directly regulates ciliary incorporation of the existing pool of already-synthesized ciliary proteins, which is not dependent on new transcription. In addition, the dominant negative mutant RanQ69L blocked ciliary entry of KAP3. Given that KAP is required for localization of KIF3A/3B to the assembly sites (Muller et al., 2005), RanGTP may control cilia length by directly regulating ciliary targeting of the heterotrimeric kinesin-II motor. This will in turn affect ciliary assembly and length maintenance due to the importance of ciliary recruitment and entry of kinesin-II motor KIF3A/3B/KAP in these processes (Engel et al., 2009; Ludington et al., 2013). Further work will determine if this is a generalized mechanism for ciliary protein import and will identify additional RanGTP-regulated ciliary proteins (cargoes) required for cilium assembly, length control, and function.

Materials and Methods

Compounds

DMSO, Importazole (IPZ, #SML0341) and Cycloheximide (C1988) were purchased from Sigma-Aldrich. Indicated concentrations and specific incubation times are used in this study.

DNA constructs

Plasmids for HA-tagged human KAP3A and KAP3B were kindly from Dr. Benjamin Allen (University of Michigan). Plasmids for wild-type Ran and point mutants RanG19V and RanT24N are a generous gift from Dr. Kristen Verhey (University of Michigan). Plasmids expressing MBP and M9M were from Dr. Yuh Min Chook (University of Texas Southwestern Medical Center). Plasmids pmCherry-C1-RanQ69L (#30309) was obtained from Addgene under the material transfer agreement. All EGFP or HA-tagged KAP3A truncations were generation by ligation-independent cloning strategy as described before (Zhu et al., 2010) and sequenced in full.

Chlamydomonas strains, mammalian cells and antibodies

Wild-type and KAP-GFP reporter strains were obtained from the *Chlamydomonas* resource center (CC-125 mt^+ and CC-4296). Strains were grown in liquid Tris-Acetate-Phosphate (TAP) liquid medium for 18-24 hours prior to experimentation. Mammalian COS-7 Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen). Human TERT-RPE cells were cultured in DMEM+F12 (1:1) (Invitrogen) containing 10% FBS. Antibodies used in this study are as follows (IF and WB are short for immunofluorescence and western blot, respectively): Mouse anti-acetylated α-tubulin (#T6793, 1:500 for IF) was from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-ARL13B (#17711-1-AP, 1:1000 for IF), Rabbit anti-Cep164 (#22227-1-AP, 1:50 for IF) and rabbit anti-IFT81 (#11744-1-AP, 1:50 for IF) were from Proteintech. Mouse anti-KAP3A (#610637, 1:20 for IF) was from BD Transduction Laboratories™. Rabbit anti-V5 (#13202, 1:1000 for WB), rabbit anti-HA (#3724, 1:100 for IF) and Rabbit anti-GFP (#2956, 1:100 for IF) were from Cell Signaling Technology.

Cell culture and transfection

COS-7 and hTERT-RPE cells were maintained in a humidified atmosphere at 37°C and 5% CO_2. Cells for transfection were seeded in an 8-well chamber slide (Lab-Tek) with 0.4 mL culture medium per well. After overnight growth, the cells became 70-80% confluent and were transfected with the corresponding plasmids using the transfection reagent FuGENE 6 (Roche)

according to the manufacturer's instructions. In normal condition, COS-7 and hTERT-RPE cells are fixed with 4% paraformaldehyde for intracellular localization assay 24 hours post-transfection. In serum starvation condition for cilium induction, hTERT-RPE cells were cultured in complete medium for 24 hours post-transfection, then followed to culture in DMEM+F12 (1:1) with 0.25% FBS for other 24 hours.

Chlamydomonas **transformation**

Electroporation transformation was used form rapid transformation of *Chlamydomonas* with the electroporator NEPA (Nepa Gene, Japan). Transformation was performed following the published protocol with some modifications (Yamano et al., 2013). The typical 4 days were necessary to perform the transformation. *Day 1*: pre-cultivation stage: the cells were grown in 5 mL TAP liquid medium for overnight culture. *Day 2*: pre-cultured cells from day 1 were transferred into a new 50 mL TAP medium in a 250 mL flask with a final OD_{730} of 0.1 (usually 1-3 mL pre-cultures added) for overnight culture with 120 rpm/25°C. *Day 3*: cells were harvested by centrifugation when the cell density reached OD_{730} of 0.3-0.4, and washed by GeneArt MAX Efficiency Transformation Reagent (Invitrogen) 3 times and resuspended in 250 μL TAP medium containing 40 mM sucrose. 1.6 μg linearized DNA was mixed with 160 μL of the cell suspension for electroporation. After electroporation, the cells were transferred into 10 mL TAP plus 40 mM sucrose for overnight culture in dim light. *Day 4*: cells were collected and plated onto 1.5% TAP-agar plate with 10 μg/mL zeocin for growth. The colonies will be visible 5-7 days later.

Immunofluorescence staining

Cells were washed with PBS twice, and then fixed with 4% paraformaldehyde in HEPES (pH 7.4) for 15 min at room temperature. Cells were washed three times with cold PBS and then incubated with 0.1% Triton X-100 in PBS (pH 7.4) for 10 min. Permeabilized cells were washed with PBS three times, and then incubated in PBST with 5% milk and 1% BSA for 1 hour at room temperature to block non-specific binding of the antibodies. Cells are incubated with diluted primary antibody in PBST with 1% BSA overnight at 4°C. After three times wash with PBS, cells are incubated with the secondary antibody in PBST with 1% BSA for 1 hour at room temperature in the dark. After washing three times with PBS, cells are mounted with ProLong Antifade mounting medium with or without DAPI, and kept at 4°C in the dark for further imaging.

Ciliary regeneration

Chlamydomonas cells were deciliated by pH shock as described before (Witman et al., 1972), and ciliary regeneration was induced in normal TAP liquid medium. After deciliation, cells were immediately treated with 10 μg/mL Cycloheximide and/or 10 μM IPZ for 60 min. For IPZ washout experiment, treated cells were washed 3 times and cultured in fresh TAP liquid medium (or with 10 μg/mL Cycloheximide). Cells were fixed with 1% glutaraldehyde for 15 min at room temperature, and cilia length was measured using the line segment tool in ImageJ.

Competing interests

The authors declare no competing or financial interests

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Figure legends

Figure 1. Kinesin-associated protein KAP3 localizes in both the cilium and nucleus. A. HA -tagged KAP3A was transfected into hTERT-RPE cells. After 24 hours transfection, cilium was induced under 0.25% serum starvation for another 24 hours. Cells were fixed with 4% paraformaldehyde (PFA) and stained with rabbit anti-HA and mouse anti-ac-α-tubulin antibodies**. B.** HA-tagged KAP3A or KAP3B were expressed in non-ciliated COS-7 cells, After

24 hour transfection, cells were fixed with 4% PFA and stained with anti-HA antibody. Cell nuclei are pseudo-colored blue, following staining with DAPI. **C**. EGFP tagged KAP3A and KAP3B were expressed in MDCK cells. **C**. EGFP tagged KAP3A and KAP3B were expressed in MDCK cells. After 24 hour transfection, cells were fixed with 4% PFA and mounted with prolong gold anti-fade mounting medium. **D.** Schematic illustration of EGFP tagged KAP3 truncations for subcellular localization assay. **E**. Mapping the domains required for nuclear localization of KAP3A in MDCK cells: a series of EGFP tagged KAP3A truncations were transfected into COS-7 cells. After 24 hours transfection, cells were fixed with 4% PFA and stained with mouse anti-HA antibody and DAPI.

Figure 2. RanGTP regulates nuclear translocation of KAP3. A. Dominant negative form of Ran blocked nuclear localization of KAP3 in COS-7 cells: GTP bound RanQ69L was co-transfected with EGFP-KAP3A into COS-7 cells. After 24 h transfection, cells were fixed with 4% PFA and stained with anti-EGFP antibody and DAPI **B.** Mapping the domains in KAP3 required for RanGTP binding: a series of EGFP tagged KAP3A truncations were transfected with GTP-locked Ran mutant Q69L into COS-7 cells. After 24 h transfection, cells were fixed with 4% PFA and stained with anti-EGFP antibody and DAPI. **C.** The effect of M9M on localization of KAP3 in COS-7 cells. EGFP tagged KAP3 co-expressed with the control MBP or M9M, and the localization of EGFP-KAP3 was examined by immunofluorescence staining with anti-EGFP antibody.

Figure 3. Armadillo repeat domain 6-9 targets KAP3 to the ciliary base. A. Schematic illustration of full-length human KAP3A and its truncated derivatives for analyzing ciliary targeting. **B.** Ciliary base localization of KAP3 truncations in hTERT-RPE cells**.** hTERT-RPE cells were transfected with various truncated constructs of KAP3. After 24 h transfection, the cells were serum starved for cilium induction for another 24 hours and fixed with 4% PFA and stained with acetylated α-tubulin (red), and nuclei are stained with DAPI (blue).

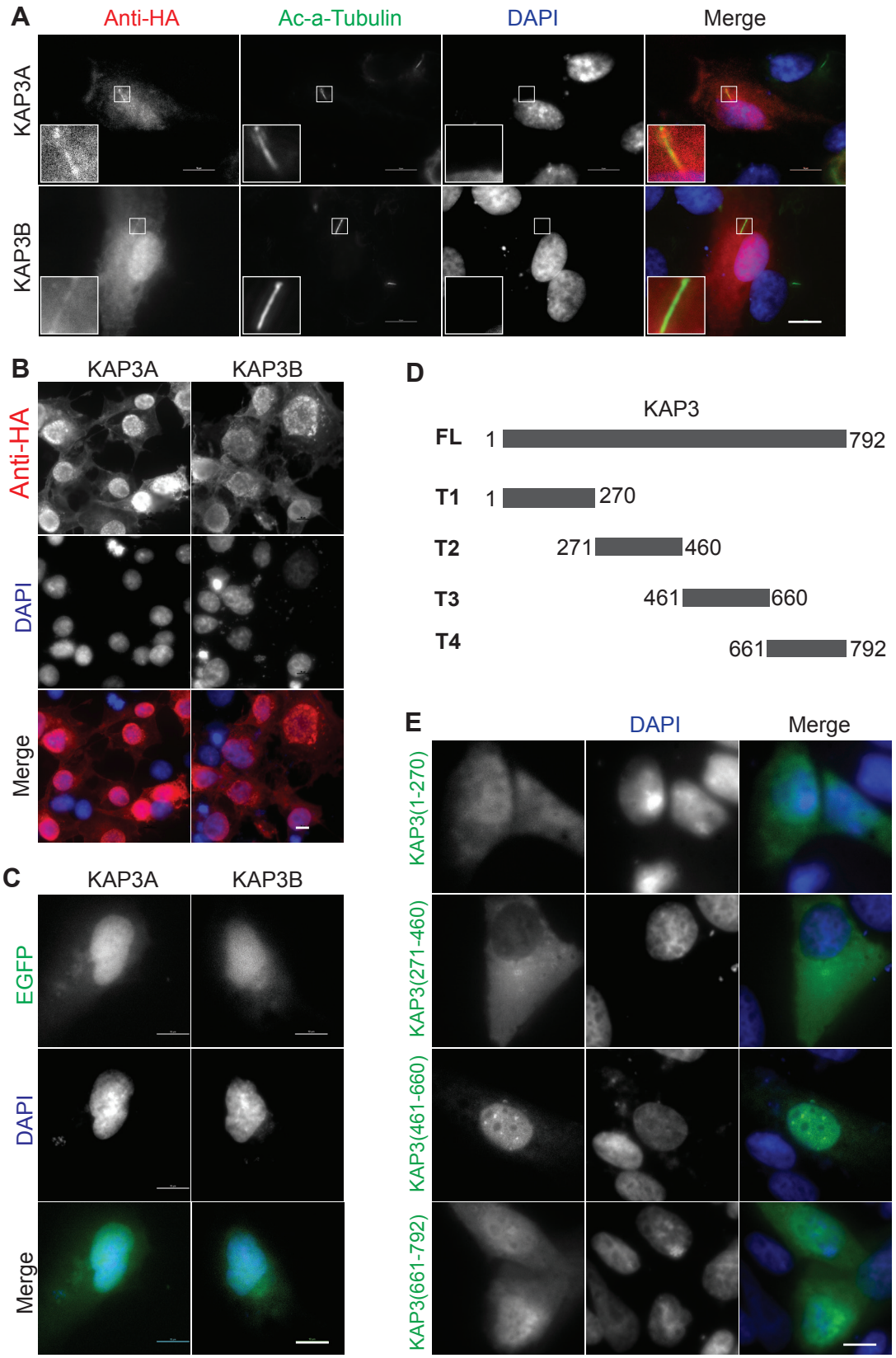
Figure 4. Dominant negative Ran mutant RanQ69L, not RanG19V and RanT24N, inhibits cilium assembly in hTERT-RPE cells. A. Subcellular localization of Ran wild type, RanQ69L, RanG19V and RanT24N in hTERT-RPE cells. Plasmids for expressing Ran wild type or point mutant were transfected into hTERT-RPE cells. Cells were fixed after 24 h transfection, and localization was visualized via immunofluorescence staining. **B.** Ran mutants blocked cilia

formation in hTERT-RPE cells**.** Different plasmids expressing point mutant Ran were transfected into hTERT-RPE cells. Cilium was induced via serum starvation 24 h post-transfection. **C.** Quantification of ciliated cells. D. RanQ69L was expressed in hTERT-RPE cells. The cells were fixed after 24 h serum depletion and labeled for acetylated α tubulin, and IFT81, Cep164 or KAP3.

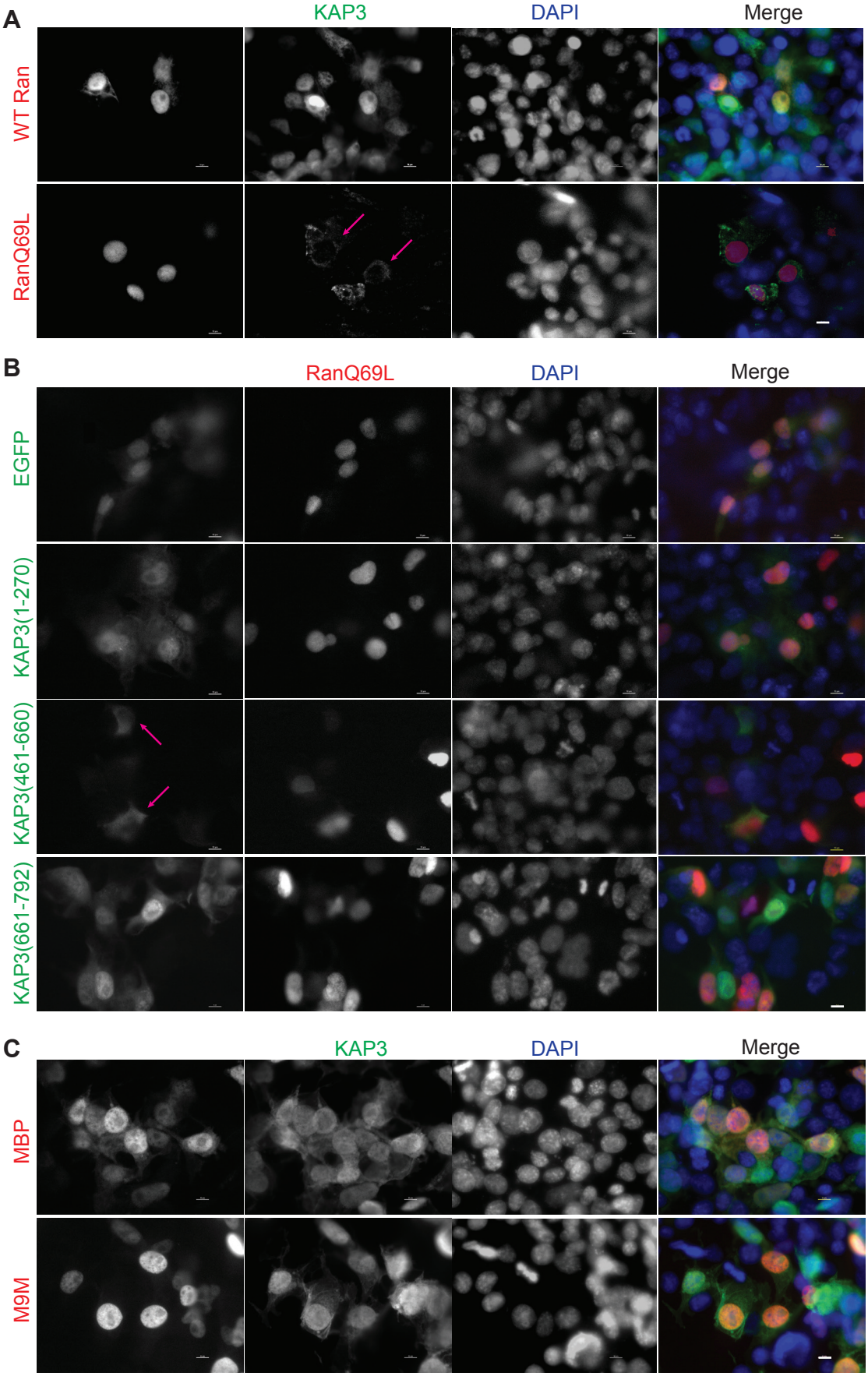
Figure 5. Disruption of RanGTP/importin β interaction shortens cilia length and blocks ciliary trafficking of KAP in *Chlamydomonas*. A. Dose dependent inhibition of cilia length by the small molecular inhibitor IPZ: CC125 wild-type cells are treated with different concentration of IPZ for 120 min, fixed with 1% glutaraldehyde, and imaged by DIC microscope at 40× magnification. **B**. Quantification of cilia length. **C** Expression of the GTP-locked Ran mutant RanQ73L shortens ciliary length in *Chlamydomonas.* pChlamy-4-RanQ73L was introduced into CC-125 cells and screened by colony PCR. The expression of RanQ73L was validated by western blot analysis via V5 antibody, and representative images for the control and RanQ73L expressing cells are shown. RanQ73L was processed correctly (green arrow) or fused with the selection marker (red arrow). **D.** Ciliary localization of KAP (pink arrows) is dramatically reduced by 10μM IPZ treatment in *Chlamydomonas* (yellow arrows). Very infrequently, a cell could be seen with some ciliary KAP localization (blue arrow). KAP-GFP reporter cells are treated with 20μM IPZ for 2 h, fixed with 100% methanol, and mounted with prolong gold anti-fade mounting medium.

Figure 6. RanGTP directly regulates ciliary protein incorporation in *Chlamydomonas* during ciliary regeneration A. Schematic representation of incorporation of already-synthesized proteins during ciliary regeneration. **A'.** Deciliated cells were treated with the combination of 10 μg/mL CHX and 10 μM IPZ for 60 min. Then IPZ, not CHX, was washed out, and cilia were regenerated for other 60 min. **B.** Wild-type CC-125 cells were deciliated, and cilia were regenerated for 1 hour in the presence of different small molecular inhibitors: 10 μg/mL CHX, 10 μM IPZ or the combination of 10 μg/mL CHX and 10 μM IPZ. Cells were fixed with 1% glutaraldehyde and imaged by DIC microscope at 40×magnification. **B'.** Schematic representation of IPZ washout in the presence of newly synthesized protein. **C.** Schematic representation of IPZ washout assay under the absence of newly synthesized protein. **C'.** Deciliated cells were treated 10 μM IPZ for 60 min and washed. Cilia were regenerated for another 60 min.

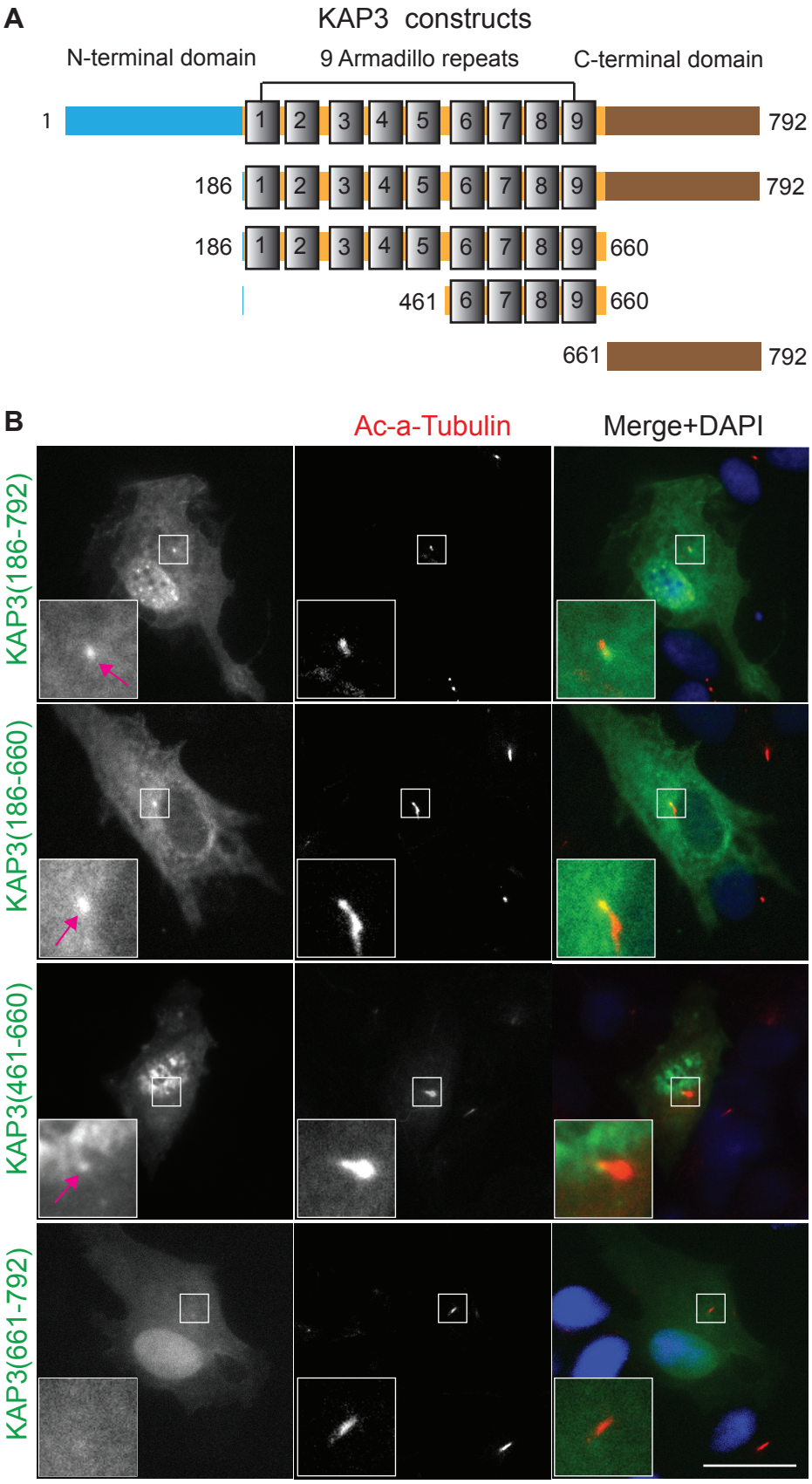
Huang et al. Figure 1



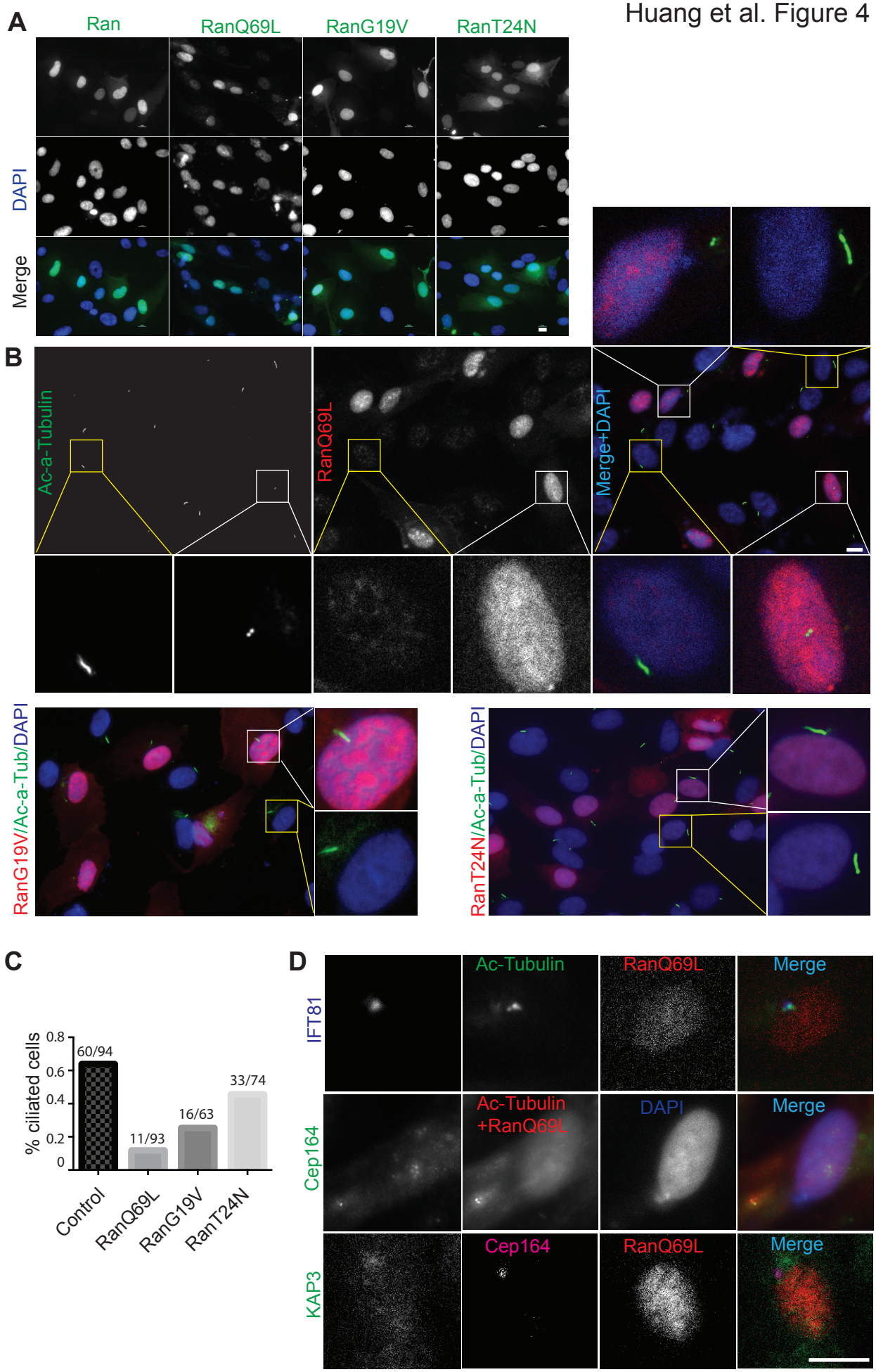
Huang et al. Figure 2



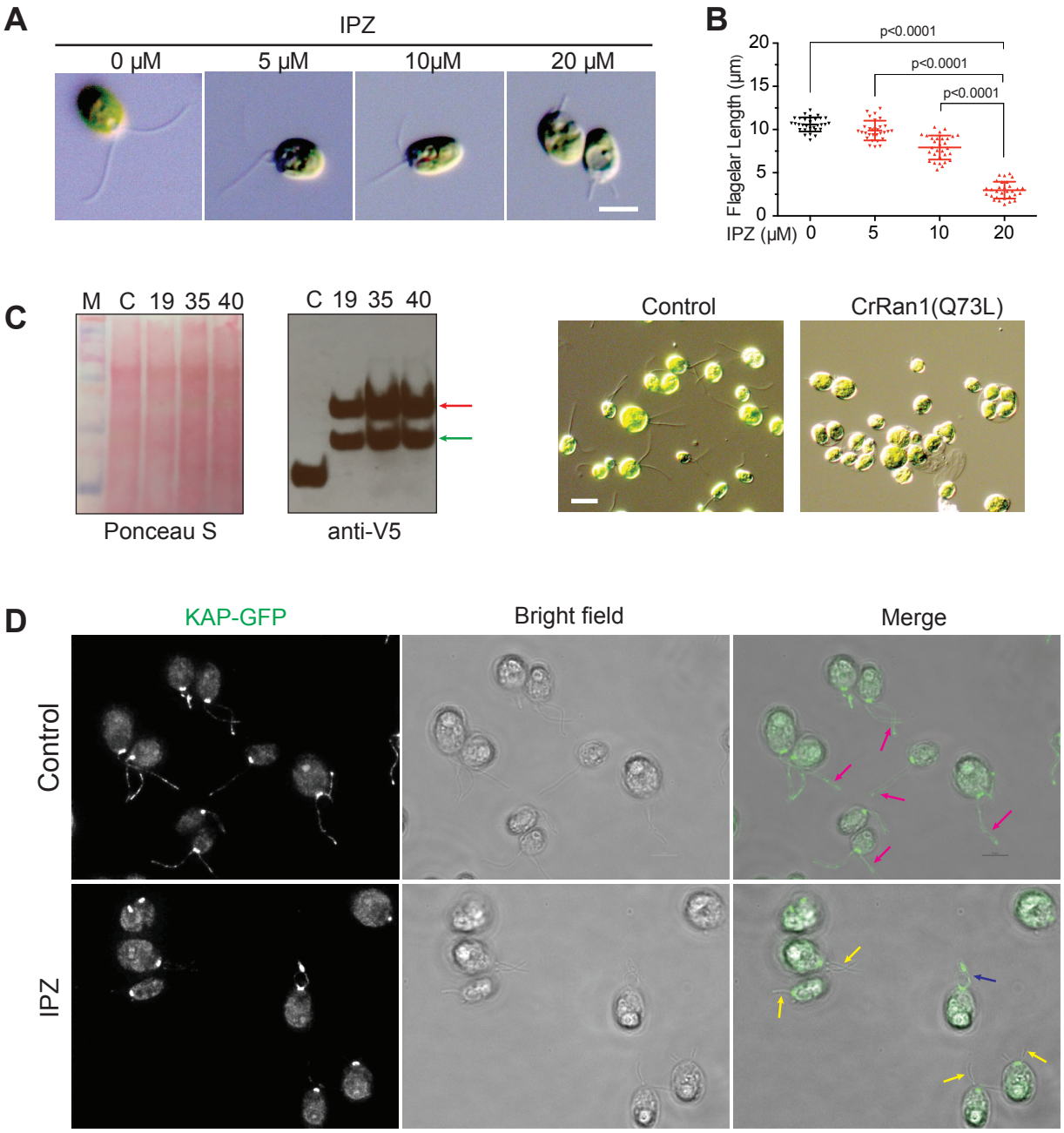
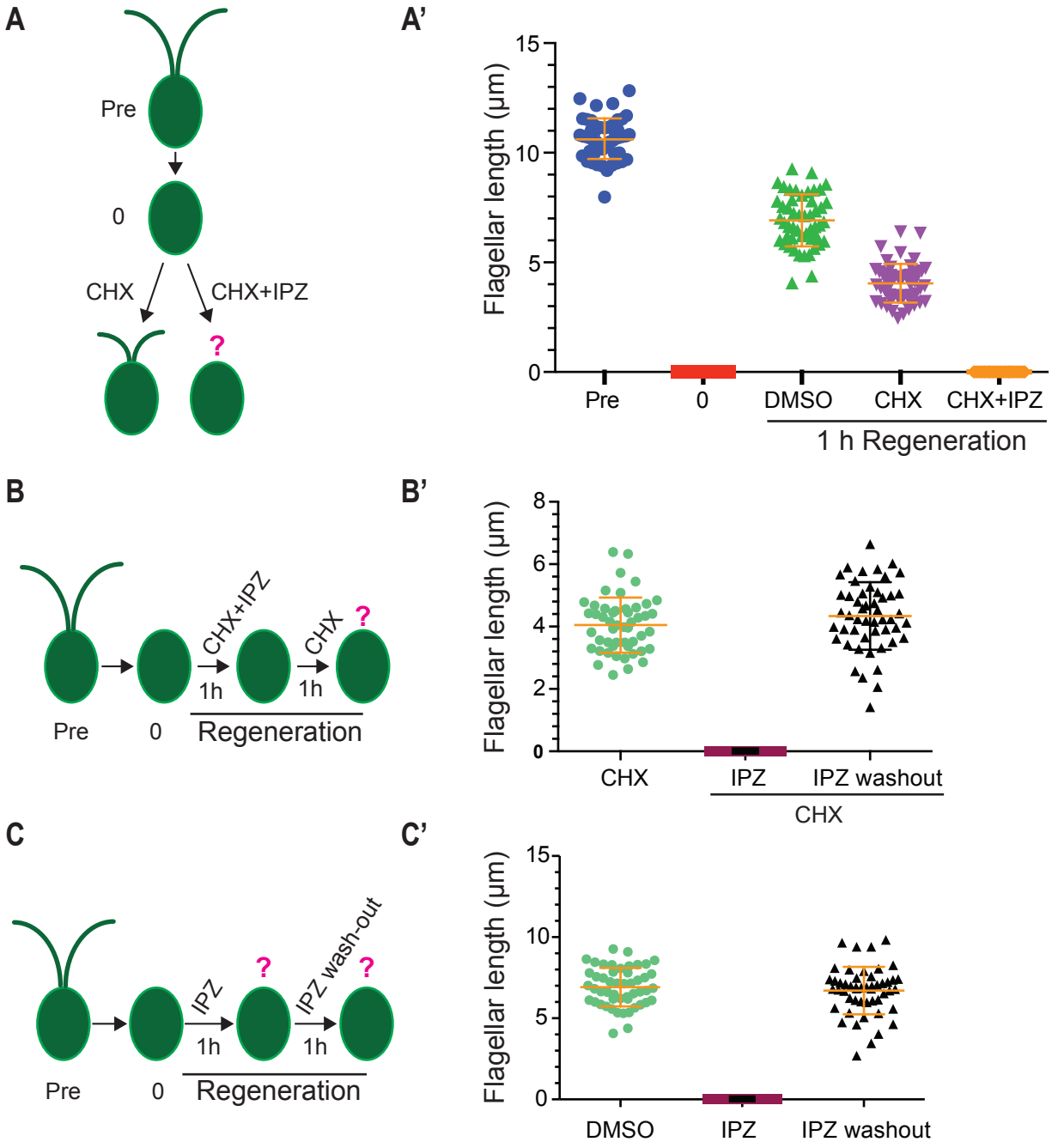
Huang et al. Figure 3



Huang et al. Figure 4



Huang et al. Figure 5



Huang et al. Figure 6