Angiomotin isoform 2 promotes binding of PALS1 to KIF13B at the base of primary cilia and suppresses ciliary elongation

Running title: Ap80 promotes PALS1 binding to KIF13B

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Abstract

The kinesin-3 motor KIF13B functions in endocytosis, vesicle transport, and regulation of ciliary length and signaling. Direct binding of the membrane-associated guanylate kinase (MAGUK) DLG1 to KIF13B's MAGUK-binding stalk (MBS) domain relieves motor autoinhibition and promotes microtubule plus end-directed cargo transport. Here we characterize Angiomotin isoform 2 (Ap80) as a novel KIF13B interactor that promotes binding of another MAGUK, the polarity protein and Crumbs complex component PALS1, to KIF13B. Live cell imaging analysis indicated that Ap80 is concentrated at the base of primary cilia and recruits PALS1 to this site, but is not itself a cargo of KIF13B. Consistent with a ciliary function for Ap80, its depletion led to elongated primary cilia and altered IGF-1 signaling in cultured mammalian cells. Our results suggest that Ap80 may specifically activate KIF13B cargo binding at the base of primary cilia to regulate ciliary length and signaling.

Introduction

Kinesin motors mediate microtubule-based transport of a variety of intracellular cargoes, including specific organelles and vesicle populations (Hirokawa and Tanaka, 2015). In humans 8 out of 45 kinesin genes code for members of the kinesin-3 family, which makes this family the largest among the human kinesin families. Kinesin-3 members are highly processive microtubule plus-end directed motors that have been implicated in transport of different types of vesicles as well as in endocytosis (Siddiqui and Straube, 2017). The kinesin-3 member KIF13B (also known as GAKIN; guanylate kinase-associated kinesin) was initially identified as a direct binding partner of the scaffold protein DLG1 in Jurkat J77 lymphocytes (Hanada et al., 2000). Subsequent studies have implicated KIF13B in many different cellular and physiological processes e.g. regulation of neuronal polarity and axon formation (Horiguchi et al., 2006, Yoshimura et al., 2010); myelination in Schwann cells and oligodendrocytes (Bolis et al., 2009, Noseda et al., 2016); cholesterol uptake and CAV1-dependent endocytosis of LRP1 in hepatocytes (Kanai et al., 2014, Mills et al., 2019); Golgi to plasma membrane trafficking of VEGFR2 in endothelial cells (Yamada et al., 2014) and of Rab6 exocytotic vesicles in HeLa cells (Serra-Marques et al., 2020); germ cell migration and Planar Cell Polarity signaling in *Xenopus laevis* (Tarbashevich et al., 2011, Ossipova et al., 2015); and regulation of ciliary CAV1 distribution and Sonic hedgehog (Shh) signaling in hTERT-immortalized retinal pigment epithelial (hTERT-RPE1; hereafter RPE1) cells (Schou et al., 2017).

KIF13B function requires tight temporal and spatial control of its motor activity, which is brought about by different binding partners that recognize specific domains in KIF13B to regulate its conformation and dimerization (Soppina et al., 2014, Siddiqui and Straube, 2017, Morthorst et al., 2018, Ren et al., 2018). The best characterized KIF13B regulator and cargo is DLG1, a Scribble polarity complex component that belongs to the membrane-associated guanylate kinase (MAGUK) homologue family. DLG1 binds directly to the MAGUK-binding stalk (MBS) domain of KIF13B, which relieves motor autoinhibition and promotes KIF13B-mediated transport of DLG1 along microtubules (Hanada et al., 2000, Asaba et al., 2003, Yamada et al., 2007, Zhu et al., 2016). Similarly, other MAGUKs including DLG4 and PALS1, a core component of the Crumbs complex required for development and maintenance of epithelial cell polarity in diverse tissues, bind directly to the KIF13B MBS domain (Zhu et al., 2016). Furthermore, we identified a physical interaction between KIF13B and NPHP4 (Schou et al., 2017), a component of the ciliary transition zone (TZ) that also associates with PALS1 and PATJ (Sang et al., 2011, Delous et al.,

2009). However, it is unknown whether an interaction between KIF13B and PALS1 is relevant in a ciliary context.

Primary cilia are microtubule-based sensory organelles present on the surface of most vertebrate cell types, and are required for the coordination of several cellular signaling pathways, including Shh (Bangs and Anderson, 2017) and receptor tyrosine kinase signaling (Christensen et al., 2017). The ciliary microtubule axoneme extends from the membrane-anchored basal body, a modified mother centriole, and is surrounded by the ciliary membrane, which is continuous with but compositionally distinct from the cell's plasma membrane. This compartmentalization is crucial for ciliary function, and is regulated by the TZ at the ciliary base (Garcia-Gonzalo and Reiter, 2017), as well as by intraflagellar transport (IFT) that via kinesin-2 and dynein 2 motors, respectively, moves trains of IFT particles with ciliary cargoes from the base to the tip of the organelle and back (Taschner and Lorentzen, 2016). Nematodes additionally express a kinesin-3 motor, KLP-6, that regulates ciliary membrane composition and function specifically in male sensory neurons by promoting release of bioactive extracellular vesicles (EVs) from the ciliary tip (Akella and Barr, 2021). Recently, we demonstrated that KIF13B displays bursts of bidirectional intraciliary movement within primary cilia of RPE1 cells and is released in EV-like particles from the ciliary tip (Dupont Juhl et al., 2021). How KIF13B intraciliary movement is regulated and the identity of ciliary cargoes of KIF13B are not known.

Here we identify and characterize Angiomotin isoform 2 (Ap80) as a new interactor of KIF13B and show that Ap80 promotes the binding of KIF13B to PALS1. Live cell imaging analysis indicated that Ap80 is concentrated at the base of primary cilia and recruits PALS1 to this site. Consistent with a ciliary function for Ap80, its depletion led to elongated primary cilia and altered IGF-1 signaling in cultured RPE1 cells. Our results suggest that Ap80 may specifically activate KIF13B cargo binding at the base of primary cilia to regulate ciliary length and signaling.

Results and discussion

KIF13B interacts with the N-terminus of Ap80. To identify KIF13B interactors we performed streptavidin pull-down assays with BirA- and GFP-tagged (BirAGFP) KIF13B constructs combined with mass spectrometry (Table 1). For this we used the deletion mutants BirAGFP-KIF13B Tail 2 (residues 607-1826) and BirAGFP-KIF13B Tail 3 (residues 752-1826), which contain and lack the MBS domain, respectively (Serra-Marques et al., 2020), reasoning that these mutants may reveal MBS-specific binding partners. We identified several significant hits that had a higher number of

peptides in the sample with the Tail 2 mutant compared to the Tail 3 mutant (Table 1). These included the already known KIF13B binding partners DLG1, together with its three known interaction partners MPP7, LIN7C and CASK (Bohl et al., 2007, Lee et al., 2002), and utrophin, a large cytoskeletal adaptor, which was shown to bind to KIF13B in a complex that mediates endocytosis of LRP1 (Kanai et al., 2014). Known binding partners of utrophin, syntrophin (Kramarcy et al., 1994) and dystrobrevins (Peters et al., 1997), were also present in the pull down. The most prominent potential novel binding partners of KIF13B, which showed a significantly stronger association with the Tail 2 compared to the Tail 3 deletion mutant, were the kinase D-interacting substrate of 220 kDa (KIDINS220, also known as ankyrin repeat-rich membrane spanning (ARMS)), a conserved transmembrane molecule implicated in different signaling pathways (Neubrand et al., 2012), and an adaptor protein belonging to the motin family, angiomotin (AMOT) (Moleirinho et al., 2014) (Table 1).

We focused on AMOT due to its reported interaction with PALS1 (Wells et al., 2006, Tan et al., 2020), which is known to associate with KIF13B (Zhu et al., 2016) and the KIF13B interactor NPHP4 (Delous et al., 2009, Schou et al., 2017). AMOT is found in two splice variants; angiomotin isoform 1 (Ap130) contains a 409 amino acid N-terminal extension not present in isoform 2, Ap80 (Ernkvist et al., 2006). We previously reported that KIF13B binds Ap80 (Schou et al., 2017), but the interaction sites were not mapped and the functional consequences of the interaction not addressed. Here, we confirmed by FLAG immunoprecipitation (IP) in HEK293T cells that FLAG-Ap80 interacts with HA-tagged full-length or motorless KIF13B (KIF13B-Δmotor-HA; residues 393-1826), but FLAG-Ap130 does not (Figure 1A). Supportively, FLAG-Ap80 was co-IPed with GFP-KIF13B (Figure 1B), and IP with different FLAG-tagged Ap80 truncations identified the first 34 residues in Ap80 as essential for KIF13B binding as a construct containing residues 1-245, which comprise the extreme N-terminus and the BAR domain region (Ap80-NB), could bind to KIF13B, but a construct spanning residues 35-245 (Ap80-B) could not (Figure 1C, D). Residues 1-245 of Ap80 are also present in Ap130, which failed to bind KIF13B (Figure 1A, C), suggesting that the KIF13B binding site in AMOT is blocked by the extended N-terminus of Ap130.

Ap80 interacts with KIF13B stalk and tail region and promotes KIF13B-PALS1 association. To map the region of KIF13B mediating the interaction with Ap80-NB, we generated plasmids coding for GFP-tagged KIF13B truncations covering most of the C-terminal stalk and tail region

(Figure 2A; (Schou et al., 2017)). Co-expression with FLAG-Ap80-NB followed by FLAG IP identified residues 561-1500 (GFP-Tail 11) as the minimal Ap80 binding site in KIF13B (Figure 2B; Figure S1A, B); reciprocal GFP IP confirmed the interaction between GFP-Tail 11 and Ap80-NB (Figure S1C). On the other hand, full length FLAG-Ap80 interacted with an even shorter KIF13B fusion, GFP-Tail 8, which contains the MBS domain but lacks residues 1327-1500 present in GFP-Tail 11 (Figure 2A,C; Figure S1D). This suggests that the KIF13B MBS domain is necessary for interaction with Ap80-NB and that residues 1327-1500 in KIF13B are also important for binding. However, the latter region is dispensable for interaction with full length Ap80. The results are consistent with our mass spectrometry data as the sequences of Tail 8 and Tail 11 are both comprised within the BirAGFP-KIF13B Tail 2 fusion capable of interacting with AMOT (Table 1; (Serra-Marques et al., 2020)).

The MBS domain of KIF13B binds directly to the guanylate kinase (GUK) domains of DLG1/DLG4 and PALS1 (Yamada et al., 2007, Zhu et al., 2016), while residues 1327-1500 contain binding sites for MARK2 (Yoshimura et al., 2010). Given that these regions of KIF13B were important for binding to Ap80, we asked if Ap80 binds KIF13B indirectly via these proteins. However, GFP-DLG1 and GFP-MARK2 did not co-IP with FLAG-Ap80, whereas HA-PALS1 did (Figure 2D; Figure S1E). We found that PALS1 binds to the C-terminus of Ap80, presumably via its PDZ domain (Ap80-C; Figure 2E; Figure S1D), while KIF13B binds to the Ap80 N-terminus (Ap80-NB; Figure 1C, D). We thus conclude that Ap80 can interact independently with both KIF13B and PALS1. Interestingly, HA-PALS1 did not co-IP with motorless KIF13B (GFP-KIF13B-Δmotor) alone, but did upon simultaneous co-expression with FLAG-Ap80 (Figure 2F). Similar results were obtained with full-length GFP-KIF13B (Figure S2A). The inability of PALS1 to associate with full-length or motorless KIF13B could be due to intramolecular interaction in KIF13B blocking access to the MBS domain. Supportively, IP using GFP- or HA-tagged KIF13B tail fragments revealed that a region spanning residues 1289-1499 interacts with the MBS region (Figure S2B, C). This result is in line with a previous study in which an interaction between a KIF13B tail fragment spanning residues 1414-1826 was shown to bind an MBS-containing fragment comprising residues 607-989 (Yamada et al., 2007). Collectively, our results suggest that Ap80 promotes association of PALS1 with KIF13B by inducing a conformational change in KIF13B that allows PALS1 to access the MBS region.

Our protein interation data is summarized in Figure 2G. First, we suggest that the interaction (a) between the NB region of Ap80 and MBS domain in KIF13B is necessary, but not

sufficient for these proteins to interact. Two additional supportive interactions can occur: one (b) between the Ap80 NB region and the region in KIF13B spanning residues 1327 to 1500, which seems important for a strong interaction between the proteins, and another (c) between the MBS domain and the C-terminal region of Ap80, likely mediated by PALS1. Binding of Ap80 to KIF13B presumably induces a conformational change in KIF13B that permits binding of the MBS domain to PALS1.

Co-localisation of Ap80, PALS1 and KIF13B at the base of primary cilia. To further explore the interaction between Ap80 and KIF13B we asked if they co-localize in cells. First, we examined the level of endogenous Ap80 and Ap130 in mouse fibroblasts (NIH 3T3 cell line) and different mouse or human epithelial cell lines, including IMCD3, HEK293T and RPE1 cells, by western blotting with AMOT antibody. We chose the RPE1 line for further analysis as it was the only one examined where we specifically detected Ap80, but not Ap130. Further, in these cells Ap80 as well as KIF13B appeared to be upregulated by high cell confluency and serum deprivation (Figure S3A), which are conditions that promote primary cilia formation (Pugacheva et al., 2007). Previously, we showed that GFP-KIF13B localizes to the base of and moves within primary cilia of RPE1 cells (Schou et al., 2017, Dupont Juhl et al., 2021). We examined, therefore, the subcellular localization of Ap80-GFP by live cell imaging in serum-deprived, ciliated RPE1 cells stably expressing the ciliary membrane marker SMO-tRFP (Lu et al., 2015). We found that Ap80-GFP is concentrated at the base of cilia and exhibits little, if any, movement within the cells (Figure 3A and Movie 1), even when co-expressed with mCherry-KIF13B (data not shown). In fixed cells, endogenous AMOT (Figure S3B) and FLAG-Ap80-NB were also detected at the base of cilia (Figure S3C). In contrast, GFP-tagged (Figure 3B) and endogenous PALS1 (Figure S3D) were mostly dispersely localized in RPE1 cells. However, and interestingly, upon co-expression with FLAG-Ap80 (Figure 3C) or GFP-Ap80 (Figure S3E) PALS1 became concentrated at the ciliary base and at cytoplasmic Ap80-positive puncta. Furthermore, in agreement with our biochemical data GFP-Ap80 and endogenous PALS1 both co-localized with KIF13B-Δmotor-HA in intracellular puncta (Figure S3F). Together, these results indicate that (1) Ap80 localizes to the base of cilia and recruits PALS1 to this site, and (2) Ap80 is not a cargo of KIF13B, but Ap80, KIF13B and PALS1 all co-localize within cells. By taking advantage of MDCK-II cells stably expressing PALS1 fused to APEX2-EGFP (PALS1-A2E) (Tan et al., 2020), we found by immunofluorescence microscopy (IFM) analysis that a pool of PALS1-A2E is localized around the ciliary base of these cells (Figure S3G). Moreover, when we

subjected the PALS1-A2E cells to APEX2-mediated proximity biotinylation for 1 min, followed by fixation and staining with fluorescently labeled streptavidin (SA-AF-566) and acetylated tubulin antibody, we observed accumulation of SA-AF-566 at the proximal region of the primary cilium (Figure S3H), further supporting that a pool of PALS1 is localized to a region near the ciliary base and that this is not limited to RPE1 cells.

Ap80 regulates ciliary length and signaling in RPE1 cells. To explore the function of Ap80 at the primary cilium we set out to knockout the *AMOT* gene (coding for both Ap80 and Ap130) in RPE1 cells using CRISPR/Cas9 methodology (Ran et al., 2013). We generated a clonal *AMOT*$^{-/-}$ line containing a homozygous 1 bp deletion in the first exon resulting in a premature stop codon at position 184-186 (Figure S4A), which led to a lack of detectable Ap80 protein (Figure 3D). Interestingly, IFM analysis of serum-deprived cells using antibodies against ciliary markers (acetylated α-tubulin and ARL13B) revealed that *AMOT*$^{-/-}$ RPE1 cells have significantly longer cilia compared to wild type cells (Figure 3E, F). The *AMOT*$^{-/-}$ cells also seemed to have fewer cilia compared to control cells, but this apparent difference was not statistically significant (Figure S4B). For technical reasons it was not possible to perform rescue analysis in the *AMOT*$^{-/-}$ cells. However, partial depletion of Ap80 in RPE1 cells using endoribonuclease-prepared small interfering RNA (esiRNA) recapitulated the long cilia phenotype observed in *AMOT*$^{-/-}$ cells (Figure S4C-E). Conversely, RPE1 cells overexpressing GFP-Ap80 had significantly shorter cilia compared to cells expressing GFP alone (Figure 3G). This was not simply due to protein over expression because the average relative expression level of GFP-Ap80 was about 5 times lower (18.2% ±2.0%, n=3) compared to that of GFP (100%, n=3), whereas the average transfection efficiencies were 25.1% ±7.2% (n=3) for GFP-Ap80 and 58.8% ±6.0% (n=3) for GFP, respectively. Increased ciliary length can lead to altered cell signaling (Broekhuis et al., 2013). Serum-deprived *AMOT*$^{-/-}$ cells displayed reduced Insulin-like Growth Factor-1 (IGF-1) signaling (Figure 3H, I), which operates via primary cilia in different cell types including fibroblasts (Zhu et al., 2009) and RPE1 cells (Yeh et al., 2013). Thus depletion of Ap80 leads to elongated cilia and reduction in IGF-1 signaling in RPE1 cells.

Conclusion

We characterized Ap80 as a new KIF13B interactor that promotes binding of KIF13B to PALS1. Ap80 does not appear to be a KIF13B cargo, judged from live cell imaging analyses, but seems to

be associated the ciliary base where it recruits PALS1 and controls ciliary length and signaling. Ap80 was previously shown to bind RICH1, a GTPase-activating protein for the Rho GTPases Cdc42 and Rac1 (Richnau and Aspenstrom, 2001), and mediate its targeting to a protein complex at epithelial tight junctions containing PALS1, PATJ and PAR3 (Wells et al., 2006). This promoted re-localization of PALS1, PATJ and PAR3 from tight junctions to recycling endosomes, thereby causing loss of tight junction integrity and induction of epithelial-mesenchymal transition (EMT) (Wells et al., 2006, Heller et al., 2010). Similarly, in endothelial cells Ap80 promoted breakdown of cellular junctions to stimulate endothelial to mesenchymal transition (EndoMT) (Bratt et al., 2005, Levchenko et al., 2004, Troyanovsky et al., 2001). Our work suggests that Ap80 may have an additional role in controlling the trafficking of PALS1 or even the entire Crumbs complex (CRB3, PALS1, PATJ, LIN7C) to the ciliary base. Supportively, components of the Crumbs complex have been detected in cilia, and CRB3 expression is required for ciliogenesis (Fan et al., 2004, Bazellieres et al., 2018).

Although more work is needed to define the precise mechanisms by which Ap80 regulates ciliary protein trafficking, length and signaling, these could include local regulation of the actin cytoskeleton, e.g. through ciliary recruitment of RICH1. Indeed, the RICH1 effector Cdc42 was localized at the base of primary cilia where it restricts axoneme length and regulates ciliary signaling through modulation of the actin cytoskeleton, and by recruiting the PAR3/PAR6/aPKC complex to this site (Drummond et al., 2018). Interestingly, Ap80 also associates physically with the PAR3/PAR6/aPKC complex (Wells et al., 2006), which was implicated in ciliogenesis (Sfakianos et al., 2007, Pruliere et al., 2011) as well as in regulation of KIF13B activity in neuronal cells through phosphorylation and inactivation of MARK2 (Yoshimura et al., 2010). In future studies it will be interesting to explore these interactions further from a ciliary perspective.

Materials and Methods

Antibodies. For western blotting, the following primary antibodies were used (catalog numbers and dilutions in parenthesis): rabbit anti-FLAG (Sigma, cat# F7425; 1:1,000), rabbit anti-GFP (Santa Cruz, cat# sc-8334; 1:1,000), rabbit anti-HA (SantaCruz, cat# sc-805; 1:500), mouse anti-DCTN1 (BD Biosciences, cat# 610474; 1:1,000), mouse anti-PALS1 (Santa Cruz, cat#sc-365411; 1:200), rabbit anti-GAPDH (Cell Signaling Technology, cat# 2118S; 1:1,000), rabbit anti-AMOT (kindly provided by Dr. Joseph Kissil, Scripps Research Institute, Jupiter, FL, USA; 1:1,000), rabbit anti-IFT88 (ProteinTech, cat# 13967-1-AP; 1:2,000). Secondary antibodies used for immunoblotting

were Horseradish Peroxidase (HRP)-conjugated swine anti-rabbit (DAKO, cat# P0399; 1:4,000) or goat anti-mouse (DAKO, cat# P0447; 1:4,000).

For IFM analysis, the following primary antibodies were used: mouse anti-FLAG (Sigma cat# F1804; 1:1,000), mouse anti-PALS-1 (Santa Cruz, cat#sc-365411; 1:50), goat anti-MYC (Abcam, cat# ab9132; 1:500), chicken anti-GFP (Abcam, cat# ab13970; 1:2,000), rabbit anti-ARL13B (ProteinTech cat# 17711-1-AP; 1:500), rabbit anti-AMOT (kindly provided by Joseph Kissil, Scripps Research Institute, Jupiter, FL, USA; 1:200), mouse anti-DCTN1 (BD Biosciences, cat# 610473; 1:500), mouse anti-acetylated α-tubulin (Sigma, cat# T7451; 1:2,000). Secondary antibodies used for IFM were all from Invitrogen and diluted 1:600: Alexa Fluor 488-conjugated donkey anti-mouse (A-21202), donkey anti-goat (A-11055), donkey anti-rabbit (A-21206) and goat anti-chicken (A11039); Alexa Fluor 568-conjugated donkey anti-mouse (A-10037), and donkey anti-rabbit (A-10042); Alexa Fluor 647-conjugated donkey anti-mouse (A-31571), donkey anti-rabbit (A-31573).

PCR, cloning procedures and plasmids. All PCR and cloning was done following standard procedures; primer sequences are listed in Table S1 and plasmids used are listed in Table S2. Plasmids encoding truncated versions of GFP-KIF13B (Tail 7, 8, 9, 11 and 12) were generated by PCR with relevant primers using pEGFP-C1-KIF13B as template (Asaba et al., 2003), followed by cloning into pEGFP-C1 (Clontech). Plasmids encoding truncated KIF13B-HA fusions (Tail 1 and 16) were generated by PCR with relevant primers using pcDNA3-KIF13B-HA plasmid (Lamason et al., 2010) as template. Plasmids encoding truncated versions of FLAG-Ap80 (-NB, -B, -C and -BC) were generated by PCR with relevant primers using pCMV-Ap80 as template, followed by cloning into pFLAG-CMV2 (Sigma). Plasmid encoding GFP-Ap80 or Ap80-GFP was generated by PCR with relevant primers using pCMV-Ap80 as template, followed by cloning into pEGFP-C1 or pEGFP-N1, respectively (Clontech). For CRISPR-Cas9 mediated knockout of AMOT, 25 nt oligos targeting exon 1 in AMOT (5'- AATACCGTGGTCCCTCCACTTGG-3') were cloned into pSpCas9(BB)-2A-Puro (PX459) using the procedure in (Ran et al., 2013). *Escherichia coli* DH10B was used for transformation and plasmid amplification. Plasmid purification was performed using NucleoBond Xtra Midi EF Kit from Macherey-Nagel. Inserts were sequenced at Eurofins MWG Operon, Ebersberg, Germany.

Cell culture and transfections. Unless otherwise stated, cells were grown at 37°C, 5% CO_2 and 95% humidity in Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% heat inactivated fetal bovine serum (FBS, Gibco) and 10 ml 1^{-1} penicillin-streptomycin (Gibco) and cell cultures were passaged every 3-4 days. Cell lines used were: HEK293T cells (laboratory stock, originally derived from American Type culture collection (ATCC), clone CRL-3216), hTERT-RPE1 cells (laboratory stock; originally derived from the immortalized hTERT-RPE1 cell line, ATCC, clone CRL-4000), hTERT-RPE1 cells stably expressing SMO-tRFP (Lu et al., 2015), swiss NIH3T3 mouse fibroblasts (laboratory stock, originally derived from ATCC clone CRL-1658), Mouse Inner Medullary Collecting Duct (IMCD3) cells (laboratory stock, originally derived from ATCC, clone CRL-2123), and PALS1-A2E MDCK-II cells (Tan et al., 2020). IMCD3 cells were grown in 45% DMEM and 45% F-12 (Ham; Sigma) with 10% FBS and 10 ml 1^{-1} penicillin-streptomycin whereas the PALS1-A2E MDCK-II cells were grown and subjected to APEX2-mediated biotinylation as described previously (Tan et al., 2020). Generation of *AMOT*$^{-/-}$ RPE1 clone was done using the CRISPR-Cas9 method described in (Ran et al., 2013) with plasmid pSpCas9(BB)-2A-Puro (PX459) encoding AMOT-specific guide RNA targeting the first exon of AMOT (5'-AATACCGTGGTCCCTCCACTTGG -3'). Screening of 91 clones by western blot analysis led to identification of a single clone with no detectable Ap80 expression. Sequencing of PCR-amplified genomic DNA from this clone revealed a 1 bp deletion in exon 1 resulting in a premature stop codon.

For plasmid transfections cells were grown to 40-50% confluency and transfections were performed using FuGene6 (Promega, E2692) according to the manufacturer's protocol. For IP experiments, HEK293T cells were grown in 10 cm diameter petri dishes and transfected with 4 µg DNA. Six hours after transfection the medium was changed to fresh growth medium and the cells were incubated for 16 hrs before harvest. For IFM experiments, RPE1 cells were grown on glass coverslips in 20 mm diameter dishes and transfected with 1 µg DNA. Six hours after transfection the medium was changed to either growth or serum-deprived medium for 16-24 hrs. For western blot experiments, RPE1 cells were grown in 60 mm diameter dishes and transfected with 2 µg DNA. Six hours after transfection the medium was changed to either growth or serum-deprived medium for 16-24 hrs.

All transfections with esiRNA (Sigma MISSION® esiRNA human AMOT cat# EHU129771) and control siRNA (5'-UAAUGUAUUGGAAUGCAUA(dTdT)-3' from Eurofins MWG Operon) were carried out as double-transfections using DharmaFECT Duo (Dharmacon).

RPE1 cells were grown to 80% confluency and transfected according to the manufacturer's manual. Six hours after transfection the medium was changed to fresh growth medium. The following day the cells were transfected again. 36 hrs later, the cells were split and an appropriate number of cells were seeded for IFM or western blot analysis. Prior to fixation or cell lysis the cells were serum-deprived for 16-24 hours.

Streptavidin pulldown assays and mass spectrometry. HEK293 cells were transfected with BirAGFP-KIF13B Tail 2 or Tail 3 constructs (designated C2 and C3, respectively, in (Serra-Marques et al., 2020)) using Polyethylenimine (PEI; Mw 2500; Polysciences) at a 3:1 PEI:DNA ratio (w/w). Cells were harvested 24 hours after transfection, by scraping the cells in ice-cold PBS and lysing cell pellets in the lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1.0% Triton X-100, and protease inhibitors; Roche). Supernatants and pellet fractions were separated by centrifugation at maximum speed for 20 minutes. Supernatants were mixed with an equal amount of Dyna M-280 Streptavidin beads (Life Technologies). Samples were incubated for 2 hours while rotating at 4°C, collected with a magnet and pellets were washed 5-7 times with the wash buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 % Triton X-100). Samples were eluted in SDS sample buffer and 30 µl of each sample was run on a 12% Bis-Tris 1D SDS-PAGE gel (Biorad) for 1 cm and stained with colloidal Coomassie dye G-250 (Gel Code Blue Stain Reagent, Thermo Scientific). Each lane was cut into 1 band, which was treated with 6.5 mM dithiothreitol (DTT) for 1 hour at 60 °C for reduction and 54 mM iodoacetamide for 30 min for alkylation. The proteins were digested overnight with trypsin (Promega) at 37°C. The peptides were extracted with acetonitrile (ACN) and dried in a vacuum concentrator. The data were acquired using an Orbitrap Q Exactive mass spectrometer. Peptides were first trapped (Dr Maisch Reprosil C18, 3 µm, 2 cm x 100 µm) before being separated on an analytical column (Zorbax SB-C18, 1.8 µm, 40 cm x 50 µm), using a gradient of 60 min at a column flow of 150 nl min^{-1}. Trapping was performed at 8 µl/min for 10 min in solvent A (0.1 M acetic acid in water) and the gradient was as follows 7-30% solvent B (0.1 M acetic acid in acetonitrile) in 31 min, 30-100% in 3 min, 100% solvent B for 5 min, and 7% solvent B for 13 min. Full scan MS spectra from m/z 350-1500 were acquired at a resolution of 35,000 at m/z 400 after accumulation to a target value of 3e6. Up to ten most intense precursor ions were selected for fragmentation. HCD fragmentation was performed at normalized collision energy of 25% after the accumulation to a target value of 5e4. MS/MS was acquired at a resolution of 17,500. In all cases nano-electrospray was performed at 1.7 kV using an in-house made gold-coated fused

silica capillary (o.d. 360 μm; i.d. 20 μm; tip i.d. 10 μm). Raw files were processed using Proteome Discoverer 1.3 (Thermo Scientific, Bremen, Germany). The database search was performed against the Swissprot human database, taxonomy (version November 2012) using Mascot (version 2.3, Matrix Science, UK) as search engine. Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as enzyme and up to two miss cleavages were allowed. Data filtering was performed using percolator, resulting in 1% false discovery rate (FDR). Additional filter was Mascot ion score >20. Raw files corresponding to one sample were merged into one result file.

Immunoprecipitation. 24 hours before IP HEK293T cells were transfected with relevant plasmids. Cells were harvested using ice cold EBC buffer (140 mM NaCl, 50 mM Tris-HCl, 0.5% NP-40, 5 mM EDTA and protease inhibitor cocktail (Roche)) and briefly sonicated prior to 20 min centrifugation at 20,000 *g*, 4°C. For FLAG, GFP and HA IP, cleared cell lysates were incubated rotating for 1 hr at 4°C with 20 µl bead slurry of Anti-FLAG (M2) conjugated beads (Sigma, cat# A2220) or 25 µl bead slurry of GFP-Trap®_A beads (Chromotek, cat# gta-20) or 20 µl bead slurry of Pierce™ Anti-HA Agarose Conjugate (Thermo Fisher Scientific, cat#26181), respectively. The immunocomplexes were washed 4 times with EBC buffer and eluted with SDS-PAGE sample buffer. The eluted proteins were then subjected to SDS-PAGE and western blotting analysis.

Protein concentration determination, SDS-PAGE and western blotting. The BioRad *DC* Protein Assay was used to determine the protein concentration of cell lysates. SDS-PAGE and western blotting was performed as described in (Schou et al., 2017).

Immunofluorescence microscopy. For IFM analysis, RPE1 cells were grown on glass coverslips, and subjected to 16-24 hrs serum-deprivation to induce ciliogenesis. Cells were washed in ice cold PBS, fixed with 4% paraformaldehyde (PFA) solution for 15 min at room temperature (RT) and cell membranes were permeabilized by incubation in 1x PBS with 0.2% (v/v) Triton X-100 and 1% (w/v) bovine serum albumin (BSA) for 12 min at RT. A blocking step of 30 min incubation at RT in 1x PBS with 2% BSA was done to avoid unspecific binding of antibodies, followed by incubation with primary antibodies over night at 4°C. After three washing steps with BSA, cells were incubated in appropriate Alexa Fluor-conjugated secondary antibodies, diluted in BSA, for 45 min at RT and nuclei were labeled with DAPI. Coverslips were mounted using 1x PBS, 90%

glycerol and 2% n-Propyl Gallate on glass slides and edges were sealed with nail polish. Fluorescence images were captured on a fully motorized Olympus BX63 upright microscope with an Olympus DP72 color, 12.8-megapixel, 4.140 x 3.096-resolution camera and with a fully motorized and automated Olympus IX83 Inverted microscope with a Hamamatsu ORCA-Flash 4.0 camera (C11440-22CU). The software used was Olympus CellSens dimension. Images were processed for publication using Adobe Photoshop version CS6. Procedures for confocal microscopy analysis of PALS1-A2E MDCK-II cells are described in (Tan et al., 2020).

Live cell imaging

Transfection and live cell imaging of RPE1 cells stably expressing SMO-tRFP were done as described previously (Dupont Juhl et al., 2021).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6 software.

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Table 1. Binding partners of the indicated BirAGFP-KIF13B constructs in HEK293T cells identified by mass spectrometry analysis.

Gene name*	Protein ID*	KIF13B Tail 2		KIF13B Tail 3	
		Unique Peptide	PSM	Unique Peptide	PSM
KIF13B	Q9NQT8	147	294	102	177
MOV10	Q9HCE1	27	27	24	24
MARK2	Q7KZI7	26	31	17	19
HSPA5	P11021	22	24	16	18
KIDINS220	**Q9ULH0**	**22**	**22**	**8**	**8**
HADHA	P40939	20	21	11	11
UTRN	**P46939**	**19**	**19**	**2**	**2**
LRPPRC	P42704	17	17	10	10
MARK3	P27448	16	18	17	18
CUL3	Q13618	16	17	12	13
DLG1	**Q12959**	**15**	**16**	**0**	**0**
AMOT	**Q4VCS5**	**14**	**15**	**0**	**0**
MARK1	Q9P0L2	12	12	6	6
HADHB	P55084	11	12	10	10
ATAD3C	Q5T2N8	11	11	10	11
SNTB2	**Q13425**	**11**	**11**	**4**	**4**
NCL	P19338	9	9	9	9
MPP7	**Q5T2T1**	**9**	**9**	**0**	**0**
KLHL12	Q53G59	8	9	7	7
OSBPL8	Q9BZF1	8	9	5	5
PPP2R1A	P30153	5	5	4	4
ILF2	Q12905	5	7	3	3
CCT6A	P40227	5	5	3	3
TCP1	P17987	5	5	3	3
HNRNPH1	**P31943**	**5**	**5**	**2**	**2**
RPS11	**P62280**	**5**	**5**	**1**	**1**
PPP2R2A	**P63151**	**5**	**6**	**1**	**1**
CCT4	P50991	4	4	4	4
HNRNPA1	P09651	4	4	4	4
PKN3	Q6P5Z2	4	4	4	4
CSNK2A1	P68400	4	4	3	3
DDX6	P26196	4	4	3	3
LRRC59	Q96AG4	4	4	2	2
LIN7C	**Q9NUP9**	**4**	**4**	**0**	**0**
PDP1	**Q9P0J1**	**4**	**4**	**0**	**0**
CASK	**O14936**	**4**	**4**	**0**	**0**
KLHL7	Q8IXQ5	3	3	2	2

FAM98A	Q8NCA5	3	3	2	2
CCT7	Q99832	3	4	1	1
AIFM1	O95831	3	3	1	1
IRS4	O14654	3	3	1	1
DDX3X	O00571	3	3	1	1
DTNB	**O60941**	**3**	**4**	**0**	**0**
FBXW11	Q9UKB1	3	3	0	0
XPR1	Q9UBH6	3	3	0	0
DTNA	**Q9Y4J8**	**3**	**3**	**0**	**0**
CEP104	O60308	3	3	0	0
GLG1	Q92896	3	3	0	0
C14orf166	Q9Y224	2	2	4	4
PSPC1	Q8WXF1	2	2	3	3
KHSRP	Q92945	2	2	2	2
PPIA	P62937	2	2	2	2
MARK4	Q96L34	2	2	2	2
ABCF1	Q8NE71	2	2	2	2
NEDD8	Q15843	2	3	1	2
PRKDC	P78527	2	2	1	1
CCT2	P78371	2	2	1	1
CCT5	P48643	2	2	1	1
XRCC6	P12956	2	2	1	1
HS2ST1	Q7LGA3	2	2	1	1
DDX21	Q9NR30	2	2	1	1
CLTC	Q00610	2	2	1	1
SLIRP	Q9GZT3	2	2	0	0
DNAJA1	P31689	2	2	0	0
DRG1	Q9Y295	2	2	0	0
KPNA2	P52292	2	2	0	0
BTBD9	Q96Q07	2	2	0	0
OSBPL5	Q9H0X9	2	2	0	0
GTF2I	P78347	2	2	0	0
COL18A1	P39060	2	2	0	0
BAG2	O95816	1	1	1	1
MOGS	Q13724	1	1	1	1
GNB2L1	P63244	1	1	1	1
CACYBP	Q9HB71	1	1	1	1
EIF3C	Q99613	1	1	1	1
TARDBP	Q13148	1	1	1	1
STAU1	O95793	1	1	1	1
EWSR1	Q01844	1	1	1	1
TTC30A	Q86WT1	1	1	1	1
DHX36	Q9H2U1	1	1	1	1
PHB	P35232	1	1	0	0

VDAC2	P45880	1	2	0	0
PAM16	Q9Y3D7	1	1	0	0
RAB10	P61026	1	1	0	0
MIB1	Q86YT6	1	1	0	0

*The highlighted genes/proteins preferentially bind the KIF13B Tail 2 over Tail 3 and are discussed in the text.

Figure legends

Figure 1. Ap80 interacts with KIF13B via its N-terminus. (**A, D**) FLAG IP of HEK293T cells co-expressing different FLAG-tagged Ap80 or Ap130 fusions and KIF13B-HA or KIF13B-Δmotor-HA. (**B)** GFP IP of HEK293T cells co-expressing FLAG-Ap80 and GFP-KIF13B. Input (in) and IP pellet (IP) fractions were subjected to SDS-PAGE and western blotting with indicated antibodies. **(C)** Diagram of full length Ap80 and Ap130 and the different truncated FLAG-Ap80 fusions generated. CC/BAR, coiled-coil and BAR domain; ABD, angiostatin binding domain; PDZ-BD, PDZ-binding domain.

Figure 2. Ap80 binds KIF13B tail region and promotes KIF13B-PALS1 association. (A) Diagram of full length and truncated KIF13B tail fusions used. KIF13B tail fusions that bind to Ap80-NB are indicated in bold. FHA, Forkhead-associated domain; MBS, MAGUK-binding stalk domain; DUF, domain of unknown function; red asterisks, MARK2 phosphorylation sites (Ser1381 and Ser1410); PRR, proline-rich region; CG, CAP-Gly domain. Redrawn from (Yoshimura et al., 2010, Soppina et al., 2014, Yamada et al., 2014, Zhu et al., 2016, Schou et al., 2017). **(B)** FLAG IP of HEK293T cells co-expressing FLAG-Ap80-NB and GFP alone (Vector) or different GFP-KIF13B tail fusions. Input (in) and IP pellet (IP) fractions were analyzed by SDS-PAGE and western blotting using indicated antibodies. **(C-E)** FLAG IP of HEK293T cells expressing the indicated fusion proteins, analyzed as in (B) using indicated antibodies. (**F**) GFP IP analysis of HEK293T cells expressing the indicated fusion proteins and analyzed as in (B) using indicated antibodies. GFP-KIF13BΔm: GFP-KIF13B-Δmotor. (**G**) Model for Ap80-KIF13B interaction. Three potential interactions between the two proteins are indicated by a, b, and c (see text for details). Note that the KIF13B N-terminus is to the right.

Figure 3. Ap80 localizes to the ciliary base and regulates ciliary length and signaling in RPE1 cells. (A) Frames of a representative time-lapse movie of an RPE1 cell co-expressing SMO-tRFP (red) and Ap80-GFP (green). The ciliary base is indicated with an asterisk in the first panel, time is in seconds. (**B**, **C**) Snapshots of RPE1 cells co-expressing SMO-tRFP (red) and PALS1-GFP (green) in the absence (**B**) or presence (**C**) of FLAG-Ap80. The ciliary base is indicated with asterisk. **(D)** Western blot analysis of WT and *AMOT*$^{-/-}$ cells using indicated antibodies. **(E)** IFM analysis of serum-deprived WT and *AMOT*$^{-/-}$ cells using antibodies against acetylated α-tubulin (AcTub, red) and ARL13B (green) to mark the cilium (arrows). DNA was stained with DAPI

(blue). **(F)** Quantification of cilia length based on data as shown in (B), with >69 cilia measured in total per condition (n=3). **(G)** Quantification of cilia length in RPE1 cells expressing GFP or GFP-Ap80, respectively; 18-35 cilia were measured per condition per experiment (n=3). **(H)** Western blot analysis of serum-deprived WT and *AMOT*$^{-/-}$ RPE1 cells treated for various times with 200 ng/ml IGF-1, using antibodies as indicated. DCTN1 serves as loading control. **(I)** Quantification of the relative levels of AKT-S^{473} phosphorylation after 30 min of IGF-1 stimulation (n=3). Data in panels F, G and I are represented as mean ± SD. Significance was determined using an unpaired, two-tailed Student's *t* test: ***, P≤0.001; ****, P≤0.0001.

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Figure 1

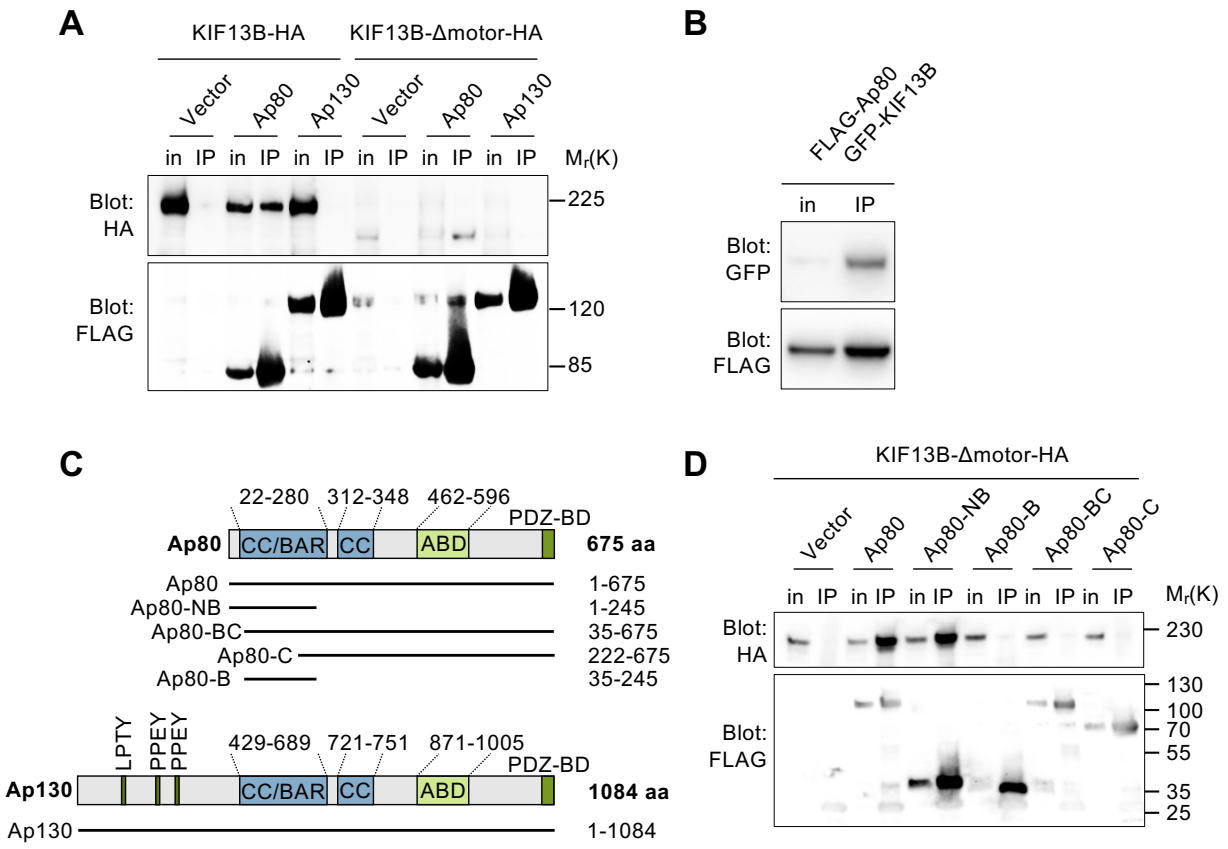
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Figure 2

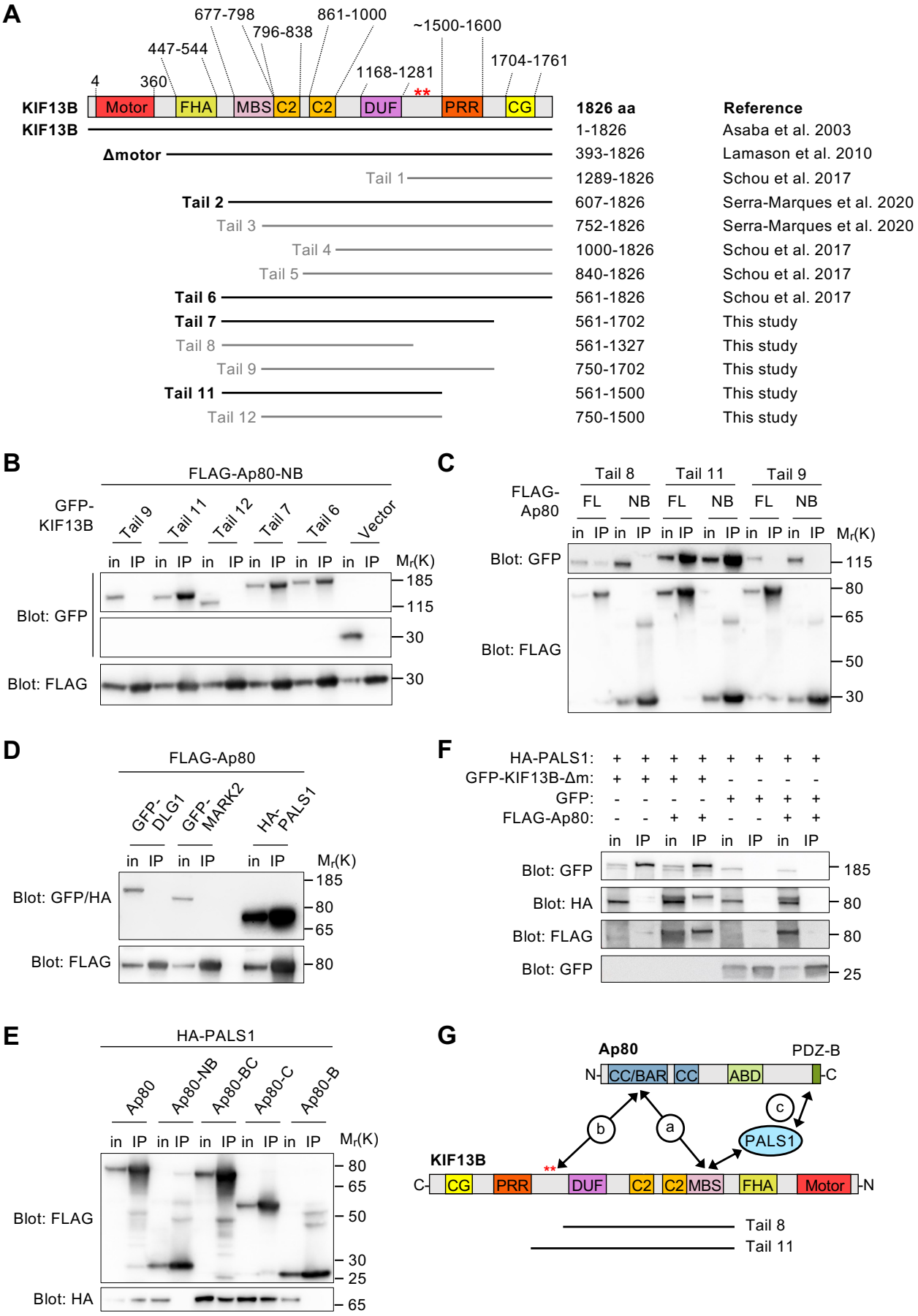
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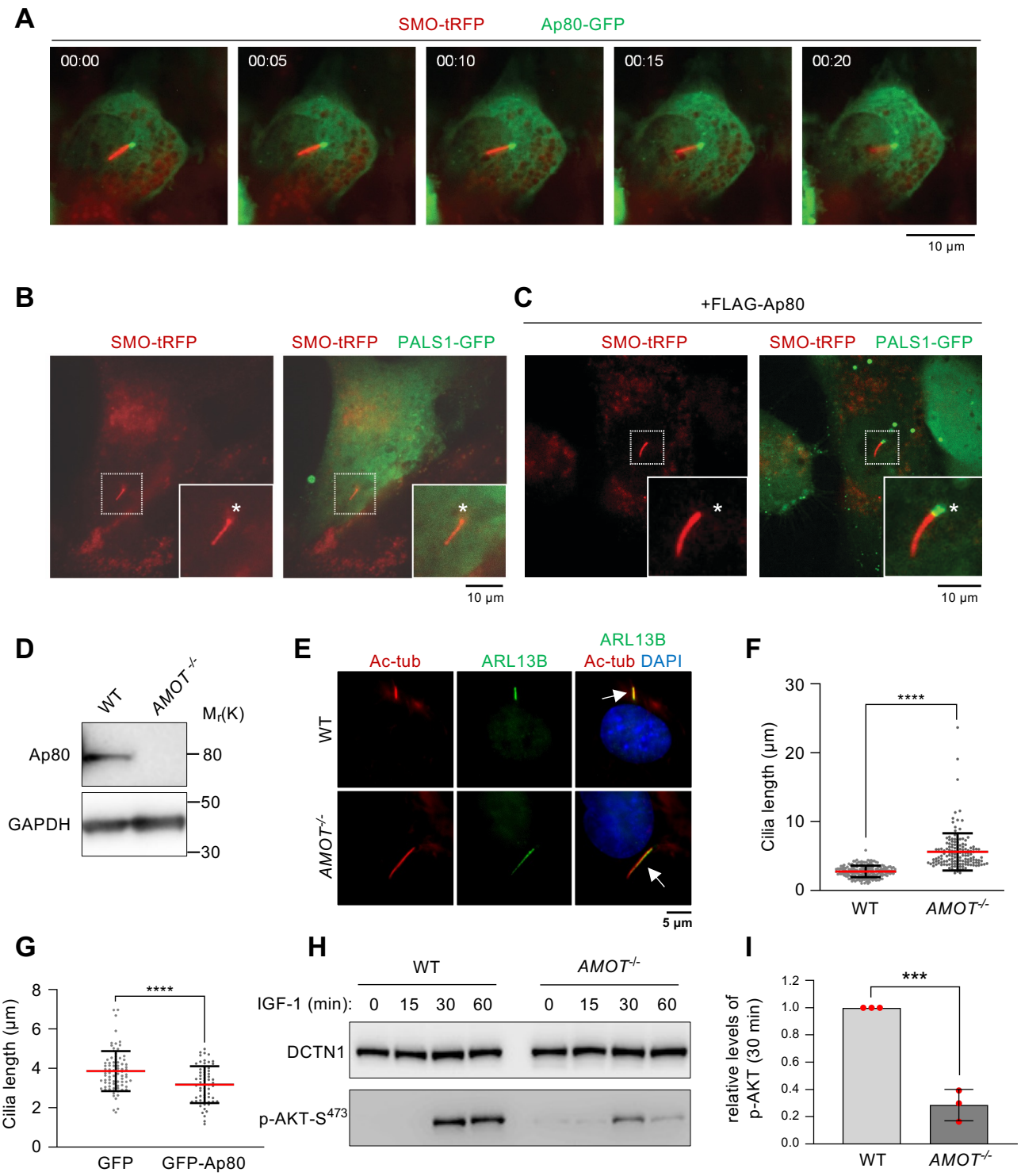
Figure 3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