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BROMI/TBC1D32 together with CCRK/CDK20 and FAM149B1/JBTS36 contributes to intraflagellar transport turnaround involving ICK/CILK1

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ABSTRACT  
Primary cilia are antenna-like organelles that contain specific proteins, and are crucial for tissue morphogenesis. Anterograde and retrograde trafficking of ciliary proteins are mediated by the intraflagellar transport (IFT) machinery. BROMI/TBC1D32 interacts with CCRK/CDK20, which phosphorylates and activates the intestinal cell kinase (ICK)/CILK1 kinase, to regulate the change in direction of the IFT machinery at the ciliary tip. Mutations in BROMI, CCK, and ICK in humans cause ciliopathies, and mice defective in these genes are also known to demonstrate ciliopathy phenotypes. We show here that BROMI interacts not only with CCRK but also with CFAP20, an evolutionarily conserved ciliary protein, and with FAM149B1/Joubert syndrome (JBTS)36, a protein in which mutations cause JBTS. In addition, we show that FAM149B1 interacts directly with CCRK as well as with BROMI. Ciliary defects observed in CCRK-knockout (KO), BROMI-KO, and FAM149B1-KO cells, including abnormally long cilia and accumulation of the IFT machinery and ICK at the ciliary tip, resembled one another, and BROMI mutants that are defective in binding to CCRK and CFAP20 were unable to rescue the ciliary defects of BROMI-KO cells. These data indicate that CCRK, BROMI, FAM149B1, and probably CFAP20 altogether regulate the IFT turnaround process under the control of ICK.

INTRODUCTION  
A variety of eukaryotic cell types use primary cilia as cellular antennae to sense and transduce mechanical signals, such as fluid flow, and biochemical signals, such as the Hedgehog (Hh) family of morphogens (Nachury and Mick, 2019; Kopinke et al., 2021). To fulfill these functions, there are specific receptors and ion channels on the ciliary membrane. The membrane and interior of cilia are distinguished from the plasma membrane and cytoplasm, respectively, by the presence of the ciliary gate comprising transition fibers and the transition zone (TZ), which together act as a permeability/diffusion barrier (Garcia-Gonzalo and Reiter, 2017; Nachury and Mick, 2019). Not only bidirectional protein trafficking along the axonal microtubules within cilia but also import and export of ciliary proteins across the ciliary gate are mediated by a large supramolecular complex called the intraflagellar transport (IFT) machinery.
(Taschner and Lorentzen, 2016; Nakayama and Katoh, 2020). Owing to the crucial roles of primary cilia in embryonic development and tissue homeostasis, mutations in the genes encoding components of the IFT machinery and the TZ result in a large heterogeneous group of disorders, collectively termed the ciliopathies, which include Bardet-Biedl syndrome (BBS) and Joubert syndrome (JBTs) (Braun and Hildebrandt, 2017; Reiter and Leroux, 2017).

The main components of the IFT machinery, often referred to as IFT trains, are the IFT-A and IFT-B complexes (Taschner and Lorentzen, 2016; Nakayama and Katoh, 2020). The IFT-B complex is composed of 16 subunits and mediates the anterograde (from ciliary base to tip) trafficking of proteins powered by the heterotrimeric kinesin-II motor, whereas the heterohexameric IFT-A complex mediates retrograde trafficking powered by the dynein-2 complex (also known as IFT dynein). In addition to these roles in intraciliary trafficking, the IFT-A complex together with the TULP3 adaptor protein mediates the import of membrane proteins across the TZ (Mukhopadhyay et al., 2010; Badgandi et al., 2017; Hirano et al., 2017; Han et al., 2019; Picariello et al., 2019; Kobayashi et al., 2021), whereas the IFT-B complex in conjunction with the BBSome, which is composed of eight BBS proteins, mediates the removal of membrane proteins from cilia (Liu and Lechtreck, 2018; Nozaki et al., 2018; Ye et al., 2018; Nozaki et al., 2019; Liu et al., 2021).

The anterograde and retrograde trafficking along the axonal microtubules entails the change in IFT direction at the ciliary tip. The turnaround process includes the remodeling of IFT trains, switching of the motor from kinesin to dynein, and loading of retrograde cargoes (Nakayama and Katoh, 2020). Consistent with this notion, cryoelectron tomographic studies revealed that Chlamydomonas anterograde and retrograde IFT trains are distinct in size and configuration (Stepanek and Pigno, 2016; Jordan et al., 2018), and a cryoelectron microscopic study suggested that the human dynein-2 complex is transported as an inactive anterograde cargo (Toropova et al., 2019). In addition, mixing of components of the IFT-A and IFT-B complexes at the tip was suggested by live-imaging analyses of the IFT trains moving in flagella/cilia of Chlamydomonas, Caenorhabditis elegans, and Trypanosoma brucei (Chien et al., 2017; Bertiaux et al., 2018; Mijalkovic et al., 2018). However, recent studies in Chlamydomonas flagella suggested that a conversion of an anterograde to retrograde trains does not involve considerable remodeling at the tip (Wingfield et al., 2021), and that the machinery at the ciliary tip is not always required for the turnaround event (Nievergelt et al., 2021).

One of the key regulators of the turnaround process is intestinal cell kinase (ICK; recently renamed as CILK1) (Fu et al., 2019; Chaya and Furukawa, 2021). ICK and its tissue-specific paralog, male germ cell-associated kinase (MAK), are remote members of the MAP kinase superfamily that contain a canonical TXY motif in the activation loop and localize at the ciliary tips by being transported via binding to the IFT-B complex (Omori et al., 2010; Chaya et al., 2014; Nakamura et al., 2020). ICK mutations in humans cause ciliopathies (Lahiry et al., 2009; Oud et al., 2016; Taylor et al., 2016), and Ick mutant mice demonstrate ciliopathy-like phenotypes (Chaya et al., 2014; Moon et al., 2014; Ding et al., 2018). As the kinase activity of ICK is essential for its function (Chaya et al., 2014; Nakamura et al., 2020), the phosphorylation of some substrates by ICK is thought to be crucial for the turnaround process. KIF3A, a motor subunit of heterotrimeric kinesin-II, was proposed to be a candidate substrate of ICK in previous studies (Chaya et al., 2014; Oh et al., 2019), but a recent study suggested that KIF3A phosphorylation is not important for ciliary function (Gailey et al., 2021). On the other hand, it has recently been shown that a phosphomimetic mutant of a C. elegans ICK orthologue can phosphorylate the IFT-B subunit IFT74 (Li et al., 2021).

Another kinase, cell cycle-related kinase (CCRK, also known as CDK20), was shown to phosphorylate ICK at the TXY motif (Fu et al., 2005; Fu et al., 2006), and in a Chlamydomonas strain defective in a CCRK orthologue (LF2), it was shown that a MAPK-related kinase (LF4) homologous to MOK/ICK/MAK does not undergo phosphorylation (Wang et al., 2019). Consistent with the role of ICK down-stream of CCRK, mutations of ICK and CCRK homologues in various organisms cause a long cilia/flagella phenotype and often increase the variation in the ciliary length of individual cells (Asleson and Lefebvre, 1998; Berman et al., 2003; Bengs et al., 2005; Burghoorn et al., 2007; Tam et al., 2007; Omori et al., 2010; Yang et al., 2013; Moon et al., 2014; Okamoto et al., 2017; Yi et al., 2018; Jiang et al., 2019; Maurya et al., 2019; Wang et al., 2019; Nakamura et al., 2020). Furthermore, mutations of CCRK and BROMI (also known as TBC1D32), which interact with CCRK (Ko et al., 2010; Noguchi et al., 2021), in humans cause ciliopathies, and CCRK and BROMI mutant mice demonstrate defects in embryonic development caused by aberrant Hh signaling (Ko et al., 2010; Adly et al., 2014; Snouffer et al., 2017; Wang et al., 2018; Alsaah and Alkuraya, 2020; Hietamäki et al., 2020). We have recently shown that not only in ICK-knockout (KO) cells but also in CCRK-KO cells derived from human telomerase reverse transcriptase-immortalized retinal pigment epithelial 1 (hTERT-RPE1) cells, the average ciliary length is longer and the variation in ciliary length is larger than in control RPE1 cells, and that excessively accumulated proteins at the bulged ciliary tips of these KO cells are eliminated via extracellular vesicles (ECVs) (Noguchi et al., 2021). It is interesting to note that the aberrant phenotypes of CCRK-KO cells were not rescued by the exogenous expression of a kinase-dead CCRK mutant or a mutant lacking a short C-terminal region responsible for BROMI binding (Noguchi et al., 2021).

In this study, we first analyzed the potential interactions of CCRK and BROMI with CAF20 (also known as FAP20, BUG22, and GTL3), as two independent interactome studies suggested that CCRK and BROMI interact directly or indirectly with CAF20 (Boldt et al., 2016; Huttlin et al., 2017), but the potential interactions have not been characterized in detail to date. We then established BROMI-KO cells and compared their phenotypes with those of CCRK-KO and ICK-KO cells and also analyzed the phenotypes of BROMI-KO cells expressing various BROMI mutants. While this study was underway, a FAM149B1-like protein in C. elegans was reported to regulate cilia homeostasis via acting upstream of orthologues of CCRK and ICK (Maurya and Sengupta, 2021), although FAM149B1 lacks a BROMI orthologue. Therefore we also investigated how FAM149B1/JBTS36 participates in the regulation of the functions of BROMI and CCRK.

### RESULTS

**Interaction of CAF20 with BROMI–CCRK**

We recently showed that CCKR/CDK20 interacts with both the N- and the C-terminal regions of BROMI/TBC1D32, and that CCRK regulates the turnaround event of the IFT machinery at the ciliary tip together with BROMI, and probably by phosphorylating ICK/CILK1 (Noguchi et al., 2021). In this study, we first analyzed the potential interactions of BROMI with CAF20 (Boldt et al., 2016; Huttlin et al., 2017); 2) CAF20 is a highly conserved protein, with 89% amino acid identity even between humans and Chlamydomonas reinhardtii (Supplemental Figure S1A) (Laligné et al., 2010); 3) CAF20 was...
identified by proteome analysis of mammalian cilia (Ishikawa et al., 2012), and FAP20 is a component of the inner junction complex that bridges protofilaments of A- and B-tubules of doublet microtubules in Chlamydomonas and Tetrahymena (Yanagisawa et al., 2014; Ma et al., 2019; Khalifa et al., 2020). We confirmed that EGFP-fused CFAP20 is evenly distributed within cilia when expressed in hTERT-RPE1 cells (Supplemental Figure S1B). 4) FAP20/BUG22 was reported to be required for ciliary stroke in Paramecium, beating of Chlamydomonas flagella, and Drosophila sperm flagella integrity (Laligné et al., 2010; Maia et al., 2014; Meng et al., 2014; Yanagisawa et al., 2014). 5) Morpholino-mediated knockdown of CFAP20 in zebrafish caused ciliary defect phenotypes, such as a curved body axis and defective heart-looping orientation (Yanagisawa et al., 2014).

When expression vectors for BROMI-EGFP and CFAP20-mCherry (mChe) were coexpressed in HEK293T cells and the lysates prepared from the transfected cells were subjected to immunoprecipitation with GST-tagged anti-GFP Nb prebound to glutathione–Sepharose 4B beads followed by SDS–PAGE and immunoblotting analysis using an anti-mChe or anti-GFP antibody, BROMI interacts with CFAP20 via its C-terminal region. Lysates of cells coexpressing EGFP-fused BROMI constructs as indicated and CFAP20-mChe were analyzed as described in (B). (D) Lysates of cells coexpressing CFAP20-mChe, CCRK-EGFP, and EGFP-fused BROMI constructs as indicated were immunoprecipitated with GST-tagged anti-mChe Nb (LaM-2 version) prebound to glutathione–Sepharose 4B beads, followed by SDS–PAGE and immunoblotting analysis using an anti-GFP or anti-mChe antibody.

FIGURE 1: Interaction of BROMI with CFAP20 and CCRK. (A) Schematic representation of the domain organizations of CFAP20, CCRK, FAM149B1, and BROMI. DUF in FAM149B1, DUF3719. (B) Direct interaction of CFAP20 with BROMI but not with CCRK. Lysates of HEK293T cells coexpressing CFAP20-mChe and EGFP-fused BROMI or CCRK, or both, were immunoprecipitated with GST-tagged anti-GFP Nb prebound to glutathione–Sepharose 4B beads followed by SDS–PAGE and immunoblotting analysis using an anti-mChe or anti-GFP antibody. (C) BROMI interacts with CFAP20 via its C-terminal region. Lysates of cells coexpressing EGFP-fused BROMI constructs as indicated and CFAP20-mChe were analyzed as described in (B). (D) Lysates of cells coexpressing CFAP20-mChe, CCRK-EGFP, and EGFP-fused BROMI constructs as indicated were immunoprecipitated with GST-tagged anti-mChe Nb (LaM-2 version) prebound to glutathione–Sepharose 4B beads, followed by SDS–PAGE and immunoblotting analysis using an anti-GFP or anti-mChe antibody.

In a previous study, we showed that BROMI (1298 amino acid protein in humans; Figure 1A) requires both its N-terminal and C-terminal regions to interact with CCRK (Noguchi et al., 2021). We then analyzed which region(s) of BROMI is required for its interaction with CFAP20 (Figure 1C). Truncation of the C-terminal 108 amino acids abolished the interaction of BROMI with CFAP20 (lane 3) as well as with CCRK, as described previously (Noguchi et al., 2021), although the resulting BROMI(1–1190) construct was unstable (lane 8) when expressed in HEK293T cells, as described previously (Noguchi et al., 2021). Truncation of the N-terminal 242 amino acids only slightly reduced the interaction of BROMI with CFAP20 (lane 5) as well as with CCRK, as described previously (Noguchi et al., 2021). Even in the presence of coexpressed CCRK, the BROMI(1–1,190) construct lacked the ability to interact with CFAP20 (Figure 1D, lane 6), whereas the N-terminal 242 amino acid truncation marginally affected the interaction of BROMI with CFAP20 (Figure 1D, lane 5). Thus the C-terminal region of BROMI encompassing the TBC
domain is required for its interaction with CCRK and CFAP20, and its N-terminal region contributes to its interaction with CCRK and probably makes a minor contribution to its interaction with CFAP20.

**BROMI-KO cells phenocopy CCRK-KO and ICK-KO cells**

We then attempted to establish BROMI-KO and CAFAP20-KO cells from hTERT-RPE1 cells using the CRISPR/Cas9 system. However, we were unable to obtain CAFAP20-KO cells probably because CAFAP20 is essential for cell survival (Blomen et al., 2015; Wang et al., 2015). When CAFAP20-EGFP was stably expressed in RPE1 cells, it was evenly distributed within cilia (Supplemental Figure S1B), consistent with the role of CAFAP20 as a component of the inner junction complex of axonemal microtubules (Yanagisawa et al., 2014; Ma et al., 2019; Khalifa et al., 2020). Intriguingly, CAFAP20-EGFP signals were reproducibly detected at the daughter centriole (Supplemental Figure S1, B–E), suggesting that CAFAP20 is also a component of centriolar triplet microtubules. It is also interesting to note that CAFAP20-EGFP signals were reproducibly detected within the nucleus (Supplemental Figure S1, B–E). This observation may be implicated by the fact that interactome studies detected interactions of CAFAP20 with FOXJ1 and RFX3, which are master regulatory transcription factors of ciliogenesis (Li et al., 2015; Boldt et al., 2016).

On the other hand, we successfully obtained cell lines with mutations in both alleles of the BROMI gene (Supplemental Figure S2, A and B). When cells were serum-deprived for 24 h to induce ciliogenesis and then immunostained for ARL13B and acetylated α-tubulin (Ac-tubulin) as markers of the ciliary membrane and axoneme, respectively, the measured ciliary length was significantly longer in BROMI-KO cells than in control RPE1 cells, as was observed for CCRK-KO and ICK-KO cells (Figure 2, A–D; see also Figure 2M). Despite its direct interaction with BROMI, the localization of CAFAP20 in the axoneme and daughter centriole was unaltered in BROMI-KO cells as well as in CCRK-KO and ICK-KO cells (Supplemental Figure S1, B–E).

We also compared the localizations of IFT88 (an IFT-B subunit) and IFT140 (an IFT-A subunit) between control cells and BROMI-KO, CCRK-KO, and ICK-KO cells. In control RPE1 cells, IFT88 was found mainly at the ciliary base and a small proportion at the tip (Figure 2E). By contrast, a substantial proportion of IFT88 was found at the ciliary tip in BROMI-KO cells (Figure 2F) as well as in CCRK-KO and ICK-KO cells. In control RPE1 cells, IFT88 was found only around the ciliary base, low levels of expression of the EGFP-fused KAP3 (also known as KIFAP3), a subunit of the anterograde motor kinesin-II (Figure 3, A–D, I), and DYNCL2L1, a subunit of the retrograde motor dynein-2 (Figure 3, E–H, J), were also significantly enriched at the ciliary tip of BROMI-KO cells as well as CCRK-KO and ICK-KO cells compared with control RPE1 cells. These observations, therefore, suggest that kinesin-to-dynein motor switching or the initiation of retrograde trafficking after the switching is at least partially impaired in the absence of BROMI, CCRK, or ICK.

We also compared the localization of exogenously expressed mChe-ICK. Our previous study showed that in CCRK-KO cells, ICK is accumulated at the ciliary tip (Noguchi et al., 2021), probably because in the absence of CCRK, ICK does not undergo phosphorylation at the TXY motif and thereby cannot participate in the turn-around event of the IFT machinery; ICK itself is a cargo of the IFT machinery (Nakamura et al., 2020). As shown in Figure 2R, mChe-ICK was enriched at the ciliary tip in CCRK-KO cells compared with control RPE1 cells (Figure 2P). In BROMI-KO cells, mChe-ICK was also enriched at the tip (Figure 2Q), suggesting that in the absence of BROMI, ICK is not functional after its transportation to the tip, as in the absence of CCRK.

Our previous studies showed that proteins accumulated at the tips of CCRK-KO and ICK-KO cilia are often eliminated via EVs (Nakamura et al., 2020; Noguchi et al., 2021), and we found that this was also the case for BROMI-KO cells. As shown in Figure 2, S and T (see also Supplemental Videos S1 and S2), the release of ARL13B-positive EVs from the tip was observed in BROMI-KO cells. These observations altogether indicate that the change in the direction of the IFT machinery at the ciliary tip is impaired in BROMI-KO cells as well as in CCRK-KO and ICK-KO cells.

Next, we compared the ciliary localizations of GPR161 and Smoothened (SMO) between BROMI-KO and control, CCRK-KO, or ICK-KO cells. GPR161 and SMO are class A and class F GPCRs, respectively, and regulate Hh signaling in negative and positive manners, respectively (Anvarian et al., 2019; Kopinke et al., 2021). In control RPE1 cells, GPR161 is present on the ciliary membrane, whereas SMO is excluded from cilia in the basal state (Figure 4, A and I). When the Hh pathway was stimulated by treatment of cells with Smoothened Agonist (SAG), GPR161 exited and SMO entered cilia (Figure 4, E and M). In BROMI-KO cells, the basal ciliary levels of GPR161 and SMO were significantly higher than those in control RPE1 cells (Figure 4, B and J); in particular, SMO was occasionally found enriched at the tip (Figure 4J). In the presence of SAG, the GPR161 level within cilia was sustained, and the SMO level was further increased (Figure 4, F and N; see also Figure 4, Q and R). Essentially the same trends in ciliary GPR161 and SMO levels in the absence and presence of SAG treatment were observed in CCRK-KO and ICK-KO cells (Figure 4, C, D, G, H, K, L, O–R). Taking into account the facts that ICK is phosphorylated by CCRK (Fu et al., 2005, 2006), and that CCRK and BROMI form a complex (Noguchi et al., 2021), the phenotypic similarities among BROMI-KO, CCRK-KO, and ICK-KO cells are consistent with the notion that CCRK regulates ICK function together with BROMI.

**BROMI requires its interactions with CCRK and probably with CFAP20 for its function**

The length of cilia of BROMI-KO cells was restored to that of control cells by the stable expression of EGFP-fused BROMI(wild type [WT]) but not EGFP alone (Figure 5, A–C; see also Figure 5P). Similarly, the accumulation of IFT88 and IFT140 at the ciliary tip was also rescued by the expression of BROMI(WT)-EGFP but not EGFP (Figure 5, F–H and K–M; see also Figure 5, Q and R). By contrast, BROMI mutants lacking the N-terminal or C-terminal region did not significantly rescue the ciliary defects. BROMI-KO cells expressing EGFP-fused BROMI(243–1298) or BROMI(1–1190) had significantly longer cilia (Figure 5, D and E) and significantly more intense staining for IFT88 (Figure 5, I and J) and IFT140 (Figure 5, N and O) at the tip than cells expressing BROMI(WT)-EGFP (see also Figure 5, P–R). Although we could not detect the clear localization of any of the expressed BROMI constructs within cilia or around the ciliary base, low levels of expression of the EGFP-fused BROMI constructs were confirmed by immunoblotting analysis of lysates of BROMI-KO cells expressing the EGFP-fused BROMI constructs using anti-GFP antibody (Figure 5S). Taken together with a previous study showing that BROMI(1–1190) lacks CCRK-binding ability, and that BROMI(243–1298) has reduced CCRK-binding ability (Noguchi et al., 2021) as well as the data of Figure 1 showing the lack of CFAP20 binding to BROMI(1–1190) but not that to BROMI(243–1298), the interactions of BROMI with CCRK and probably with CFAP20 are expected to be required for its function.
FIGURE 2: Cilia elongation and accumulation of IFT components at the ciliary tip in BROMI-KO cells. (A–L) Control RPE1 cells (A, E, I), the BROMI-KO cell line #BROMI-2-17 (B, F, J), the CCRK-KO cell line #CCRK-2-4 (C, G, K), and the ICK-KO cell line #ICK-4-6 (D, H, L) were cultured under serum-starved conditions for 24 h to induce ciliogenesis and triple immunostained for ARL13B, acetylated α-tubulin, and γ-tubulin (A–D); IFT88, ARL13B, and FOP (recently renamed as CEP43) (E–H); or IFT140, ARL13B, and FOP (I–L). Scale bars, 5 µm. (M) Ciliary lengths of the cells in (A)–(D) were measured and expressed as scatter plots. (N, O) Relative staining intensities of IFT88 and IFT140 at the ciliary tips and base in the cells shown in (E)–(H) and (I)–(L), respectively, were estimated, and the intensity ratios of tip/(tip + base) were expressed as scatter plots. The total number of cells analyzed (n) are indicated. The triangles indicate the means. Statistical significances were calculated using one-way ANOVA followed by the Dunnett's multiple comparison test. (P–R) Control RPE1 cells (P), BROMI-KO cells (Q), and CCRK-KO cells (R) stably expressing mChe-ICK were serum-deprived for 24 h and immunostained for IFT88 and ARL13B+FOP. Scale bar, 5 µm. (S, T) The release of ECVs from the ciliary tips of the BROMI-KO cells stably expressing ARL13B(ΔGD)-EGFP. Time-lapse images of Supplemental Videos S1 and S2 are shown. Scale bars, 5 µm.
Interactions of FAM149B1 with BROMI–CFAP20 and with CCRK

While this study was in progress, a *C. elegans* study reported that the FAM149B1-like protein XBX-4 may act via a pathway involving CCRK and ICK orthologues (DYF-18 and DYF-5) to regulate cilia homeostasis (Maurya and Sengupta, 2021), although there is no direct orthologue of BROMI in the *C. elegans* genome. On the other hand, previous case studies reported that homozygous mutations in FAM149B1 cause JBTS-associated diseases in humans, resulting in abnormal ciliary phenotypes such as tip accumulation of IFT-B and dysregulation of the Hh signaling (Shaheen et al., 2019; Siegert et al., 2021), and an interactome study suggested direct or indirect interactions of FAM149B1 with BROMI and CFAP20 (Huttlin et al., 2017). However, as shown in Figure 6A, coimmunoprecipitation analysis showed that the interaction of BROMI with FAM149B1 was relatively weak (lane 3). We then analyzed the FAM149B1–BROMI interaction in the presence of CFAP20, and found that the interaction of FAM149B1 with BROMI was substantially enhanced in the presence of coexpressed CFAP20 (compare lane 5 with lane 3), although FAM149B1 did not demonstrate a direct interaction with CFAP20 alone (lane 4).

FAM149B1 has a domain of unknown function 3719 (DUF3719) in its N-terminal region (Figure 1A), and homozygous mutations in this domain resulting in premature termination (p.Gln118Hisfs*20, p.Lys119Ilefs*18, and p.Gln147*) were found to cause JBTS-associated diseases (Shaheen et al., 2019; Siegert et al., 2021). When the FAM149B1 protein was divided into the DUF3719-containing region, FAM149B1(1–179), and the remaining region, FAM149B1(180–582) (see Figure 1A), the latter retained the ability, although reduced, to interact with BROMI–CFAP20 (Figure 6B; compare lanes 3 and 4 with lane 2), indicating that the non-DUF3719 region of FAM149B1 mainly contributes to its interaction with BROMI–CFAP20. This was somewhat unexpected, as the non-DUF3719 region is not conserved between *C. elegans* and vertebrates (Maurya and Sengupta, 2021),
although there is no BROMI orthologue in *C. elegans*. Although the C-terminal nonconserved region of FAM149B1 is predicted to be intrinsically disordered (see Discussion), it did not nonspecifically interact with EGFP alone (Supplemental Figure S3).

We then analyzed whether CCRK can interact indirectly with FAM149B1 via BROMI–CFAP20. To this end, TagBFP (tBFP)-tagged CCRK was coexpressed with FAM149B1-mChe in the presence or absence of coexpressed BROMI-EGFP and/or CFAP20-EGFP in HEK293T cells, and lysates from the cells were subjected to immunoprecipitation with GST-fused anti-mChe Nb (the LaM-2 version) prebound to glutathione Sepharose 4B beads. As shown in Figure 6C, CCRK substantially coprecipitated with FAM149B1 in the presence of BROMI

- CFAP20 (lane 5) and moderately in the presence of BROMI alone (lane 3). The amount of BROMI

+ CFAP20 coprecipitated with FAM149B1 was greater in the presence of coexpressed CCRK than in its absence (compare lane 5 with lane 6), suggesting that the interaction of FAM149B1 with BROMI–CFAP20 is enhanced in the presence of CCRK. Intriguingly, a low but substantial amount of CCRK was coprecipitated with FAM149B1 even in the absence of coexpressed BROMI–CFAP20 (lane 2) or in the presence of coexpressed CFAP20 alone (lane 4), suggesting a direct interaction of FAM149B1 with CCRK. This was also unexpected, as to our knowledge, no interactome studies to date have suggested an interaction between FAM149B1 and CCRK.

We then analyzed whether the DUF3719 region or the non-DUF3719 region of FAM149B1 interacts directly with CCRK under the prediction that the conserved DUF3719 region is important for this interaction. As shown in Figure 6D, however, the non-DUF3719 region (residues 180–582) was found to interact with CCRK (lane 4). Thus the non-DUF3719 region of FAM149B1 is mainly responsible for its interactions with both CCRK and BROMI (Figure 6E). In the AlphaFold Protein Structure Database (Tunyasuvunakool et al., 2021), the non-DUF3719 region is predicted to be unstructured, suggesting that this region has the potential to interact with a variety of proteins as “an intrinsically disordered region.”

### Ciliary defects of FAM149B1-KO cells resemble those of BROMI-KO, CCRK-KO, and ICK-KO cells

We next established FAM149B1-KO cells from hTERT-RPE1 cells (Supplemental Figure S2, C and D) and analyzed their phenotypes. FAM149B1-KO cells had longer cilia and demonstrated substantial accumulation of IFT88 at the ciliary tip compared with control RPE1 cells. This accumulation of GPR161 and SMO within cilia of BROMI-KO cells. (A–P) Control RPE1 cells (A, E, I, M), BROMI-KO cells (B, F, J, N), CCRK-KO cells (C, G, K, O), and ICK-KO cells (D, H, L, P) were serum-starved for 24 h and further incubated for 24 h without (−SA; A–D, I–L) or with (+SA; E–H, M–P) 200 nM SAG. The cells were triple immunostained for GPR161 (A–H), ARL13B, and FOP or for SMO (I–P), Ac-tubulin, and γ-tubulin. Scale bars, 5 µm.

(Q, R) The relative ciliary staining intensities of GPR161 and SMO shown in (A–H) and (I–P), respectively, were estimated and expressed as scatter plots. The total number of cells analyzed (n) is indicated. Differently colored dots represent three independent experiments, and triangles are means of individual experiments. Horizontal lines and error bars are means and SDs, respectively, of the three experiments. Statistical significances among multiple cell lines were calculated using one-way ANOVA followed by the Dunnett’s multiple comparison test.

![FIGURE 4](https://repository.kulib.kyoto-u.ac.jp)
FIGURE 5: BROMI requires its interaction with CCRK and probably with CFAP20 for its functions. (A–O) Control RPE1 cells (A, F, K), or BROMI-KO cells stably expressing EGFP (B, G, L), or EGFP-fused BROMI(WT) (C, H, M), BROMI(243–1,298) (D, I, N), or BROMI(1–1,190) (E, J, O) were serum-deprived for 24 h and triply immunostained for Ac-tubulin, ARL13B, and γ-tubulin (A–E); IFT88, ARL13B, and FOP (F–J); or IFT140, ARL13B, and FOP (K–O). Scale bars, 5 µm. Note that the EGFP epifluorescence was quenched when the cells were fixed with methanol. (P) Ciliary lengths of the cells in (A)–(E) were measured and expressed as scatter plots. The total number of cells analyzed (n) are indicated. The triangles indicate the means. (Q, R) Relative staining intensities of IFT88 and IFT140 at the ciliary tips and base in the cells shown in (F)–(J) and (K)–(O), respectively, were estimated, and the intensity ratios of tip/(tip+base) were expressed as scatter plots. Differently colored dots represent three independent experiments, and triangles are means of individual experiments. Horizontal lines and error bars are means and SD, respectively, of the three experiments. Statistical significances were calculated using one-way ANOVA followed by the Dunnett’s multiple comparison test. (S) Lysates prepared from BROMI-KO cells stably expressing EGFP (lane 1) or EGFP-fused BROMI(WT) (lane 2), BROMI(243–1,298) (lane 3), or BROMI(1–1,190) (lane 4) were processed for immunoblotting analysis using an anti-GFP or anti-β-tubulin antibody.
cells (Figure 7, A and B; see also Figure 7, F and G); this is in line with the observation of fibroblasts from a patient with JBTS-like symptoms with the homozygous FAM149B1 mutation (Shaheen et al., 2019). Stable expression of FAM149B1(WT)-EGFP in FAM149B1-KO cells restored normal ciliary length and eliminated abnormal IFT88 accumulation at the tip (Figure 7C; see also Figure 7, F and G). As in BROMI-KO and CCRK-KO cells (Figure 2Q, R), stably expressed mChe-ICK was substantially enriched at the ciliary tip of FAM149B1-KO cells compared with control RPE1 cells (Figure 7, H and I). CFAP20 localization to the ciliary axoneme and daughter centriole was unaltered in FAM149B1-KO cells (Supplemental Figure S1F, G), consistent with the observation in the C. elegans

FIGURE 6: Interactions of FAM149B1 with BROMI and CCRK. (A) Direct interaction of FAM149B1 with BROMI but not with CFAP20. Lysates of HEK293T cells coexpressing FAM149B1-mChe and EGFP-fused BROMI or CFAP20, or both, were immunoprecipitated with GST-tagged anti-mChe Nb (LaM-2 version) prebound to glutathione–Sepharose 4B beads, followed by SDS–PAGE and immunoblotting analysis using an anti-EGFP or anti-mChe antibody. (B) FAM149B1 interacts with BROMI via its non-DUF3719 region. Lysates of cells coexpressing mChe-fused FAM149B1 constructs, as indicated, and EGFP-fused BROMI and CFAP20 were analyzed as described in (A). (C) Indirect and direct interactions of FAM149B1 with CCRK. Lysates of cells coexpressing mChe-fused FAM149B1 constructs as indicated, and CCRK-tBFP, and combinations of BROMI-EGFP and CFAP20-EGFP as indicated were immunoprecipitated with GST-tagged anti-mChe Nb (LaM-2) followed by SDS–PAGE and then subjected to immunoblotting analysis using an anti-tBFP antibody that recognizes tBFP, an anti-GFP antibody, or an anti-mChe antibody. (D) FAM149B1 interacts with CCRK via its non-DUF3719 region. Lysates of cells coexpressing mChe-fused FAM149B1 constructs as indicated and CCRK-EGFP were analyzed as described in (A). (E) A model of the interactions among BROMI, CCRK, FAM149B1, and CFAP20 predicted from this study. The coloring of the individual proteins is the same as shown in Figure 1A.
FAM149B1-KO cells phenocopy BROMI-KO, CCRK-KO, and ICK-KO cells. (A–E) Control RPE1 cells (A) and FAM149B1-KO cells (B) and those stably expressing EGFP-fused FAM149B1(WT), FAM149B1(1–179), or FAM149B1(180–582) were cultured under serum-starved conditions for 24 h and immunostained for IFT88 and ARL13B+FOP. Scale bars, 5 µm. (F) Ciliary lengths of the cells shown in (A)–(E) were measured and expressed as scatter plots. (G) Relative staining intensities of IFT88 at the ciliary tip and base in the cells shown in (A)–(E) were estimated, and the intensity ratio of tip/(tip + base) was expressed as scatter plots. The total number of cells analyzed (n) are indicated. The triangles indicate the means. Statistical significances were calculated using one-way ANOVA followed by the Dunnett’s multiple comparison test. (H, I) Control RPE1 cells (H) and FAM149B1-KO cells (I) stably expressing mChe-ICK were serum-deprived for 24 h and immunostained for IFT88 and ARL13B+FOP. Scale bar, 5 µm. (J) Lysates prepared from FAM149B1-KO cells (lane 1) and those stably expressing EGFP-fused FAM149B1(WT) (lane 2), FAM149B1(1–179) (lane 3), or FAM149B1(180–582) (lane 4) were processed for immunoblotting analysis using an anti-GFP or anti-β-tubulin antibody. Asterisks indicate the positions of nonspecific bands of unknown origin.

FIGURE 7: FAM149B1-KO cells phenocopy BROMI-KO, CCRK-KO, and ICK-KO cells. (A–E) Control RPE1 cells (A) and FAM149B1-KO cells (B) and those stably expressing EGFP-fused FAM149B1(WT), FAM149B1(1–179), or FAM149B1(180–582) were cultured under serum-starved conditions for 24 h and immunostained for IFT88 and ARL13B+FOP. Scale bars, 5 µm. (F) Ciliary lengths of the cells shown in (A)–(E) were measured and expressed as scatter plots. (G) Relative staining intensities of IFT88 at the ciliary tip and base in the cells shown in (A)–(E) were estimated, and the intensity ratio of tip/(tip + base) was expressed as scatter plots. The total number of cells analyzed (n) are indicated. The triangles indicate the means. Statistical significances were calculated using one-way ANOVA followed by the Dunnett’s multiple comparison test. (H, I) Control RPE1 cells (H) and FAM149B1-KO cells (I) stably expressing mChe-ICK were serum-deprived for 24 h and immunostained for IFT88 and ARL13B+FOP. Scale bar, 5 µm. (J) Lysates prepared from FAM149B1-KO cells (lane 1) and those stably expressing EGFP-fused FAM149B1(WT) (lane 2), FAM149B1(1–179) (lane 3), or FAM149B1(180–582) (lane 4) were processed for immunoblotting analysis using an anti-GFP or anti-β-tubulin antibody. Asterisks indicate the positions of nonspecific bands of unknown origin.

xbx-4 mutant (Maurya and Sengupta, 2021). Thus FAM149B1-KO cells phenocopy BROMI-KO, CCRK-KO, and ICK-KO cells with respect to their ciliary defects (Figure 2) and are likely to be impaired with respect to the turnaround process at the tip.

When the N-terminal [FAM149B1(1–179)] or C-terminal [FAM149B1(180–582)] construct was expressed in FAM149B1-KO cells, cilia did not recover to the normal length as in the case of FAM149B1(WT) expression (Figure 7, C–E; see also Figure 7F). However, somewhat unexpected was that the expression of FAM149B1(1–179) in FAM149B1-KO cells significantly rescued the defect in IFT88 accumulation at the tip similarly to the FAM149B1(WT) expression (Figure 7G). Immunoblot analysis of cell lysates confirmed the expression of the FAM149B1 constructs (Figure 7J). Although we do not know the exact reason for this observation, it suggests that the conserved DUF3719 region plays some role in ciliary protein trafficking independently of CCRK and BROMI.
DISCUSSION

Mutations in BROMI, CCRK, ICK, and FAM149B1 in humans are known to cause ciliopathies, and those in mice cause ciliopathy-like phenotypes with impaired Hh signaling (Lahiry et al., 2009; Adly et al., 2014; Oud et al., 2016; Taylor et al., 2016; Snouffer et al., 2017; Shaheen et al., 2019; Alsaheen and Alkuraay, 2020; Hietamäki et al., 2020; Siegert et al., 2021). In previous studies, we showed that ICK undergoes IFT-mediated trafficking to the ciliary tip where it regulates the IFT turnaround event in a manner dependent on its phosphorylation cycle involving CCRK (Nakamura et al., 2020; Noguchi et al., 2021).

In this study, we showed that BROMI interacts not only with CCRK but also with CAF20, which is a conserved axonemal protein, and with FAM149B1, a protein in which its mutations cause JBTS-associated diseases. We also found that FAM149B1 interacts directly with CCRK as well as with BROMI. CCRK-KO, BROMI-KO, and FAM149B1-KO cells were found to demonstrate very similar ciliary defects to ICK-KO cells, including abnormal cilia elongation and the accumulation of components of the IFT machinery at the ciliary tip. In addition, ICK itself was accumulated at the tip in these KO cells. These observations indicate that interactions of BROMI with CCRK and probably with CAF20 are crucial for the turnaround process that is controlled by ICK. The data also suggest that FAM149B1 participates in the CCRK–ICK pathway via directly interacting with both BROMI and CCRK. By elucidating the direct interactions among these components and demonstrating the similarity among cells defective in any of these components, the present study, in conjunction with previous studies on ICK and CCRK (Nakamura et al., 2020; Noguchi et al., 2021), expands on the previous studies in C. elegans suggesting that orthologues of CCRK, ICK, and Ds have regulatory roles in IFT homeostasis in the same pathway (Yi et al., 2018; Maurya et al., 2019; Maurya and Sengupta, 2021), although C. elegans lacks a BROMI orthologue. The direct interaction of FAM149B1 with CCRK suggests that the FAM149B1 orthologue can regulate the CCRK orthologue in absence of a BROMI orthologue. In this context, it is also noteworthy that a CCRK orthologue (LF2) in Chlamydomonas forms a complex with LF1 and LF3 (Tam et al., 2007), and if I1, I2, I3, and if4 mutant strains demonstrate elongated flagella (Asleson and Lefebvre, 1998), although LF1 and LF3 homologues are not present in mammals and Chlamydomonas lacks BROMI and FAM149B1 homologues. Thus the core kinases, CCRK and ICK/MAK/MOK, are likely to undergo fine tuning by species-specific modulators.

The common defects found in ICK-KO, CCRK-KO, BROMI-KO, and FAM149B1-KO cells are likely to reflect the impaired function of ICK at the ciliary tip. However, we previously showed that CCRK is localized around the base of cilia (Noguchi et al., 2021), but is not detectable within or at the tip of cilia (Ko et al., 2010; Noguchi et al., 2021). In this context, it is of note that C. elegans DVF-18 as well as DVF-5 were reported to be in the distal segments of sensory cilia in one study (Yi et al., 2018), whereas in another study both were found in the proximal regions in WT animals (Maurya et al., 2019). On the other hand, consistent with our observations regarding the localization of ICK, DVF-5 was enriched in the distal regions of cilia in dVF-5 and xba-4 mutants (Maurya et al., 2019; Maurya and Sengupta, 2021). In addition, we were unable to show in this study the distinct ciliary localization of BROMI and FAM149B1, although a low level of C. elegans XB4-4 was found in the distal region and underwent IFT movement in only a subset of cilia, suggesting that it can be transported via IFT (Maurya and Sengupta, 2021). Furthermore, proteomic analysis of cilia demonstrated that BROMI is a ciliary protein (Ishikawa et al., 2012).

Therefore where and how CCRK phosphorylates/activates ICK in conjunction with BROMI and FAM149B1 are important issues. One possible scenario is that ICK is phosphorylated by CCRK at the base of cilia under the control of BROMI and/or FAM149B1 and is then transported to the ciliary tip via binding to the IFT machinery (Nakamura et al., 2020). As CCRK/CDK2 is a remote member of the CDK family (Malumbres, 2014), BROMI and/or FAM149B1 may play roles as cyclins via directly interacting with CCRK. Given that FAM149B1, but not BROMI, is evolutionarily conserved, and that FAM149B1 can interact directly with CCRK, it is possible that FAM149B1 is the major regulator of CCRK/CDK20 function, similar to cyclins, and that BROMI acts as an adaptor between them to strengthen their interaction (see Figure 6E). In this context, it is an interesting issue whether BROMI and/or FAM149B1 affect the localization of CCRK. However, attempts to determine whether there is a difference in CCRK localization between control cells and BROMI-KO or FAM149B1-KO cells have so far been unsuccessful because the CCRK-EGFP signals at the ciliary base in control cells are very weak as described previously (Noguchi et al., 2021). Another possibility is that CCRK (as well as BROMI and FAM149B1) can transiently enter cilia and phosphorylate ICK, which in turn regulates the turnaround event at the tip, as a cilia proteome study identified BROMI as a ciliary protein (Ishikawa et al., 2012).

We also showed here that CAF20 interacts with BROMI, and enhances the interactions of BROMI with CCRK and FAM149B1, although we were unable to elucidate the physiological relevance of the interactions involving CAF20, as we could not establish CAF20-KO cells probably owing to its essential role in cell survival (Blomen et al., 2015; Wang et al., 2015). In addition to its role as an integral component of the ciliary axoneme (Yanagisawa et al., 2014; Ma et al., 2019; Khalifa et al., 2020), CAF20 is also found in the nucleus (Supplemental Figure S1, B–G) and was found to interact with master regulators of ciliogenesis in two independent interactome studies (Li et al., 2015; Boldt et al., 2016). Thus this relatively compact protein may play a variety of roles associated with cilia.

MATERIALS AND METHODS

Request a protocol through Bio-protocol.

Plasmids, antibodies, reagents, and cell lines
cDNAs for human CAF20 (NM_013242.3) and FAM149B1 (NM_173348.2) were obtained from a cDNA library by PCR amplification. Human ICK cDNA was kindly provided by Takahisa Furukawa (Osaka University) (Chaya et al., 2014). Expression vectors for BROMI, CAF20, FAM149B1, CCRK, and ICK and their deletion and point mutants and vectors for the production of lentiviruses expressing them are listed in Supplemental Table S1. Several of the vectors were constructed in our previous studies (Hamada et al., 2018; Nakamura et al., 2020; Noguchi et al., 2021). Antibodies used in this study are listed in Supplemental Table S2. GST-tagged anti-GFP Nb and anti-mChe Nb (the LaM-2 version) prebound to glutathione–Sepharose 4B beads were prepared as described previously (Katoh et al., 2015; Katoh et al., 2018; Ishida et al., 2021). SAG and Polyethylenimine Max were purchased from Enzo Life Sciences and PolySciences, respectively. HEK293T cells and hTERT-RPE1 cells were obtained from RIKEN BioResource Research Center (Catalogue No. RBC2202) and American Type Culture Collection (Catalogue No. CRL-4000), respectively.

Coimmunoprecipitation analyses
Coimmunoprecipitation analyses were performed based on the procedures described previously for the visible immunoprecipitation
Establishment of BROMI-KO and FAM149B1-KO cells

Disruption of the BROMI and FAM149B1 genes in hTERT-RPE1 cells by the CRISPR/Cas9 system using homology-independent DNA repair was carried out as described previously (Kato et al., 2017), with some modifications (Okazaki et al., 2020; Fujisawa et al., 2021). Briefly, single-guide RNA (sgRNA) sequences targeting the human BROMI/TBC1D32 or FAM149B1 gene (see Supplemental Table S3) were designed using CRISPOR (Haessler et al., 2016). Double-stranded oligonucleotides for the target sequences were inserted into the all-in-one sgRNA expression vector, pHiFiCas9-2 × sgRNA (Addgene 162277) (Fujisawa et al., 2021). hTERT-RPE1 cells seeded on a 12-well plate to approximately 1.5 × 10^5 cells, and the next day they were transfected with the all-in-one vector and the donor knock-in vector, pDonor-tBFP-NLS-Neo(universal) (Addgene 80767) (Kato et al., 2017), using X-tremeGENE9 Reagent (Roche Applied Science). After selection of the transfected cells in the presence of G418 (600 µg/ml), cells were isolated using an S1800 cell sorter (SONY) at the Medical Research Support Center, Graduate School of Medicine, Kyoto University. Genomic DNA extracted from the isolated cells was analyzed by PCR using GoTaq Master Mixes (Promega) and PrimeSTAR GXL Premix Fast (Takara Bio) and three sets of primers (Supplemental Table S3) to distinguish the following three states of integration of the donor knock-in vector: forward integration (Supplemental Figure S2, A and C, b, b′), reverse integration (c, c′), and no integration with a small indel (a, a′). The disruption of both alleles of BROMI and FAM149B1 was confirmed by direct sequencing of the PCR products.

The ICK-KO cell line #ICK-4-6 and the CCRK-KO cell line #CCRK-2-4 were established as described previously (Nakamura et al., 2020; Noguchi et al., 2021).

Preparation of cells stably expressing EGFP-fused BROMI, FAM149B1, and CFAP20 constructs

Lentiviral vectors for the expression of various BROMI and FAM149B1 constructs and a CFAP20 construct were prepared as described previously (Takahashi et al., 2012). Briefly, pRRLsinPPT-EGFP-N-BROMI or its mutant, pRRLsinPPT-EGFP-N-FAM149B1 or its mutant, or pRRLsinPPT-EGFP-N-CFAP20 was transfected into HEK293T cells along with the packaging plasmids (pRSV-REV, pMD2.g, and pMDLg/pRRE [Thomas et al., 2009]) kindly provided by Peter McPherson, McGill University. Culture media were replaced 8 h after transfection, and those containing viral particles were collected at 24, 36, and 48 h after transfection. The collected media were passed through a 0.45-µm filter and centrifuged at 32,000 × g at 4°C for 4 h. The precipitates containing lentiviral particles were resuspended in Opti-MEM (Thermo Fisher). The preparation of lentiviral vectors for the ICK constructs was as described previously (Nakamura et al., 2020; Noguchi et al., 2021). The lentiviral suspension was added to the culture medium of hTERT-RPE1 cells or KO cell lines and used for subsequent analyses after a 24-h incubation.

Immunofluorescence analysis and live-cell imaging

hTERT-RPE1, BROMI-KO, FAM149B1-KO, CCRK-KO, and ICK-KO cells were cultured in DMEM/F-12 (Nacalai Tesque) supplemented with 10% FBS and 0.348% sodium bicarbonate. To induce ciliogenesis, the cells were grown on coverslips to 100% confluence and serum-starved for 24 h in DMEM/F-12 containing 0.2% bovine serum albumin.

Unless otherwise stated, immunofluorescence analysis was performed as described previously (Noguchi et al., 2021; Zhou et al., 2022). Briefly, cells on coverslips were fixed with 3% paraformaldehyde for 5 min at room temperature and permeabilized with 100% methanol for 5 min at −20°C (for experiments shown in Figures 2, P–R; 3, A–H; 4, A–P; and 7, A–E, H and I; and Supplemental Figure S1, B–G), fixed and permeabilized with 100% methanol for 5 min at −20°C (for experiments shown in Figures 2, A–L; and 5, A–O), and washed three times with phosphate-buffered saline. The fixed/permeabilized cells were blocked with 10% FBS, incubated sequentially with primary antibody (anti-EGFP, anti-mCherry, anti-ARL13B or Ac-tubulin within cilia using a segmented line tool in the ZEN 3.1 imaging software (Carl Zeiss). For the correction of local background intensity, the ROI was duplicated and set to a nearby region. Statistical analyses were performed using JMP Pro 16 software (SAS Institute).

Live-cell imaging to observe the release of ECVs from cilia was performed as described previously (Nakamura et al., 2020; Kobayashi et al., 2021). Briefly, BROMI-KO cells expressing EGFP-fused ARL13B(AGD) were serum-starved for 24 h on a glass-bottom culture dish (MatTek) and observed under an A1R-MP confocal laser-scanning microscope (Nikon). Time-lapse images were acquired sequentially every 5 min and analyzed using NIS-Elements imaging software (Nikon).

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