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Ciliary and non-ciliary functions of Bardet-Biedl Syndrome proteins

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Dean:

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Annotation

The present thesis is written in a cumulative way and consists of the following publications:

Patnaik SR, Farag A, Volz AK, **Brücker L**, Schneider S, Kretschmer V, May-Simera HL (2020): Tissue dependent differences in Bardet-Biedl Syndrome gene expression. *Biol Cell*; 112(2):39-52 (Publication I)

Patnaik SR, Kretschmer V, **Brücker L**, Schneider S, Volz AK, Oancea-Castillo LDR, May-Simera HL (2019): Bardet-Biedl Syndrome proteins regulate cilia disassembly during tissue maturation. *Cell Mol Life Sci*; 76(4):757-775 (Publication II)

Brücker L, Becker SK, Harms G, Parsons M, May-Simera HL: The actin-bundling protein Fascin-1 modulates ciliary signalling. (Manuscript I)

Brücker L, Kretschmer V, May-Simera HL (2020): The entangled relationship between cilia and actin. Review. *IJBCB*; 129: 105877 (Publication III)

Manuscript I was submitted in February 2022 at *Journal of Molecular Cell Biology* and is currently being revised. Additional data not included in this manuscript are shown in section 3.3. Publication III represents a literature review on the topic of primary cilia and actin. Contribution to the Publications I and II and Manuscript I are described in detail in the supplementary data of this thesis.

Contribution to further publications

The following publication is not part of this thesis but has also been contributed to:

Nguyen VTT, **Brücker L**, Volz AK, Baumgärtner JC, Dos Santos Guilherme M, Valeri F, May-Simera H, Endres K (2021): Primary cilia structure is prolonged in enteric neurons of 5xFAD Alzheimer's disease model mice. *Int J Mol Sci*; 22: 13564

Contribution to conferences

Parts of this thesis have been presented on the listed conferences in form of a presentation or poster:

Brücker L, Volz AK, May-Simera HL (2021): Forschungsupdate zu Zilien und Zellmigration. Presentation. National meeting of the German BBS patient society, Essen, Germany

Brücker L, Parsons M, May-Simera HL (2019): Cilia protein regulation of actin dynamics. Poster. 2019 ASCB/EMBO Meeting, Washington D.C., USA

Brücker L (2019): Cilia – the navigation system of the cell. Presentation. 4th Interdisciplinary Symposium, Mainz, Germany

Brücker L & Volz AK (2019): DNA – Der Baustein des Lebens. Presentation. National meeting of the German BBS patient society, Essen, Germany

Brücker L, Patnaik SR, May-Simera HL (2018): Ciliary regulation of Wnt signalling molecules during cell migration. Poster. International Meeting of the German Society for Cell Biology (DGZ), Leipzig, Germany

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1. Introduction

Primary cilia are microtubule-based organelles important for various aspects of cell and tissue homeostasis. Similar to signalling antennae, they transmit and receive cues from and to other cells and regulate several intracellular signalling pathways. Thus, defects in primary cilia and ciliary development are associated with numerous human genetic disorders, collectively termed ciliopathies. Besides regulating cilia development, maintenance and trafficking, ciliary proteins have been suggested to coordinate other important functions inside the cell such as cell cycle progression (Delaval et al., 2011; Wood et al., 2012), proteasomal degradation (Gerdes et al., 2007; Kudryashova et al., 2005; Liu et al., 2014), DNA damage response (O'Regan et al., 2007), transcriptional regulation (Gascue et al., 2012), intracellular trafficking (Finetti et al., 2009; Leitch et al., 2014; May-Simera et al., 2015; Yen et al., 2006), and regulation of the cytoskeleton and cell migration (Hernandez-Hernandez et al., 2013; J. Kim et al., 2010; May-Simera et al., 2016; Yin et al., 2009). The current work aims to obtain a deeper understanding of the ciliary and non-ciliary functions of ciliary proteins to expand our knowledge of the complex molecular mechanisms underlying human ciliopathies.

1.1. Primary Cilia

Cilia are highly conserved cell organelles that can be found on many eukaryotic cell types. They are required for locomotion of cells such as ciliates or sperm cells, for fluid-flow over membranes for example in the respiratory epithelium, or for the exchange of signals and thus communication between cells. Primary cilia are found singularly on cells, whereas motile cilia can be abundant in large numbers per cell. Since they cannot move, primary cilia are important for inter- and intracellular communication of cells, thus often involved in organ and tissue development (Anvarian et al., 2019). Although motile cilia are structurally different from primary cilia as they require more stability to enable movement, both cilia types are able to receive and transduce cellular signals and are thus also involved in the regulation of different intracellular signalling pathways (Satir and Christensen, 2007). Some cells can have very specialised primary cilia such as photoreceptor cells in the mammalian retina (May-Simera et al., 2017).

1.1.1. Primary ciliogenesis

Primary cilia are anchored to the cell via the basal body, originally derived from the mother centriole, which consists of nine microtubule triplets organised in a ring-like formation (Fig. 1). Upon exit of the cell cycle, the mother centriole undergoes various stages of maturation and docks at the plasma membrane. As opposed to the extracellular way, the intracellular process of ciliogenesis (visualised in Fig. 1) is initiated via docking of preciliary vesicles at the distal end of the mother centriole (Schmidt et al., 2012; Wu et al., 2018). The ciliary vesicle is formed via fusion of distal appendage vesicles, further remodelling and maturing the mother centriole (Lu et al., 2015; Sorokin, 1968). Via extension of the inner microtubule doublets (the A- and B-tubule) of the basal body within the sheath of the ciliary vesicle, the nascent primary cilium is formed, which docks at the plasma membrane (Sánchez and Dynlacht, 2016). Distal appendages anchor the ciliary basal body at the plasma membrane, whereas subdistal appendages are needed to connect it to the intracellular microtubule network (Huang et al., 2017). Focal adhesion complexes further attach the basal body to the actin cytoskeleton (Antoniades et al., 2014). After docking at the plasma membrane, ciliary proteins and components of the intraflagellar transport machinery (IFT) are recruited to the cilium via distal appendages which enable elongation of the ciliary axoneme (Yang et al., 2018). Thus, the ciliary axoneme protrudes from the cell surface and is able to receive and transduce signals.

The thickness of the axoneme reduces concomitantly from the base to the ciliary tip which is accompanied by migration of microtubules into the axonemal centre, thus the axonemal microtubule structure dissolves from the traditional ring-like formation (Kiesel et al., 2020). Additionally, some of the B-tubules are not elongated towards the ciliary tip and often terminate within the first third of the axoneme (Kiesel et al., 2020). The microtubules provide structure and stability for the ciliary axoneme and serve as a track for the intraflagellar transport machinery. This machinery consists of specialised IFT trains that are, together with dynein and kinesin motor proteins, required for bidirectional transport of proteins and vesicles into and out of the cilium, consequently enabling its assembly and disassembly (Nozaki et al., 2019; Wei et al., 2012). The IFT trains are organised at the transition zone between the basal body and the axoneme, which serves as a size-dependent diffusion barrier regulating protein cargo into and out of the cilium (Kee et al., 2012). The specifically regulated trafficking of signalling receptors and proteins into and out of the axoneme maintains the ciliary function as a specialised signalling hub.

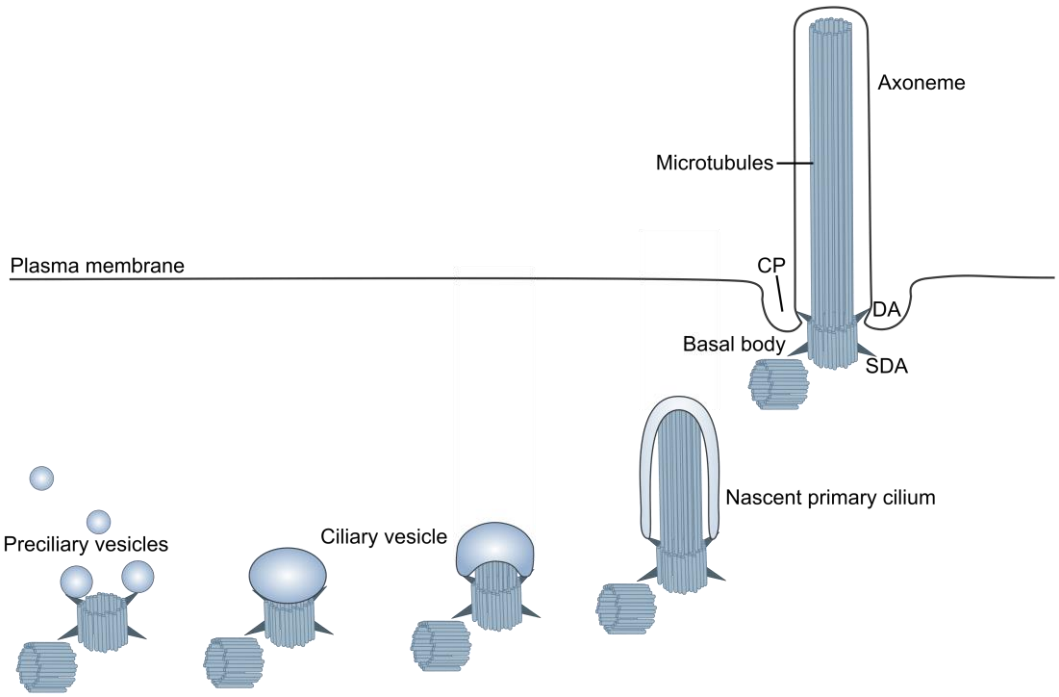

Fig. 1: The intracellular process of ciliogenesis.
During ciliogenesis, preciliary vesicles dock at the mother centriole, inducing its maturation. Fusion of the preciliary vesicles result in the formation of the ciliary vesicle. Elongation of microtubule doublets lead to the development of a nascent primary cilium that docks at the plasma membrane via distal appendages (DA) and emerges from the cell surface. The mature primary cilium consists of the basal body, which is anchored to the plasma membrane via distal appendages and to the microtubule network via subdistal appendages (SDA), and the axoneme, extending from the basal body. The ciliary pocket (CP) is formed via invagination of the membrane.

1.1.2. Actin during ciliogenesis

Although the primary cilium is a predominantly microtubule-based structure, recent research indicates a prominent role for F-actin and actin-associated proteins in ciliary development and maintenance. Actin structures are important for various aspects of cell homeostasis such as division, proliferation and migration. These structures include focal adhesions, stress fibres, lamellipodia, and filopodia (Fig. 2 A). Focal adhesions are required to anker the cell onto the substrate via actin regulators such as vinculin and paxillin (Humphries et al., 2007). They are further connected to stress fibres assembled by RhoA, containing anti-parallel F-actin bundles and extending all over the cell to form a stable actin network (Ridley and Hall, 1992). Within lamellipodia, found at the leading edge of the cell, a dynamic branched F-actin network is formed via the Arp2/3 actin nucleators and Rho GTPases, that cyclically builds up and collapses, moving the cell forward (Lai et al., 2008; Schaks et al., 2021). Microspikes called

filopodia are formed within lamellipodia. These structures move beyond the edge of lamellipodia to sense and seek out the surrounding environment. Instead of branched actin networks, filopodia contain a parallelized structure of dynamic F-actin filaments bundled via the actin regulator Fascin-1 (Pfisterer et al., 2020).

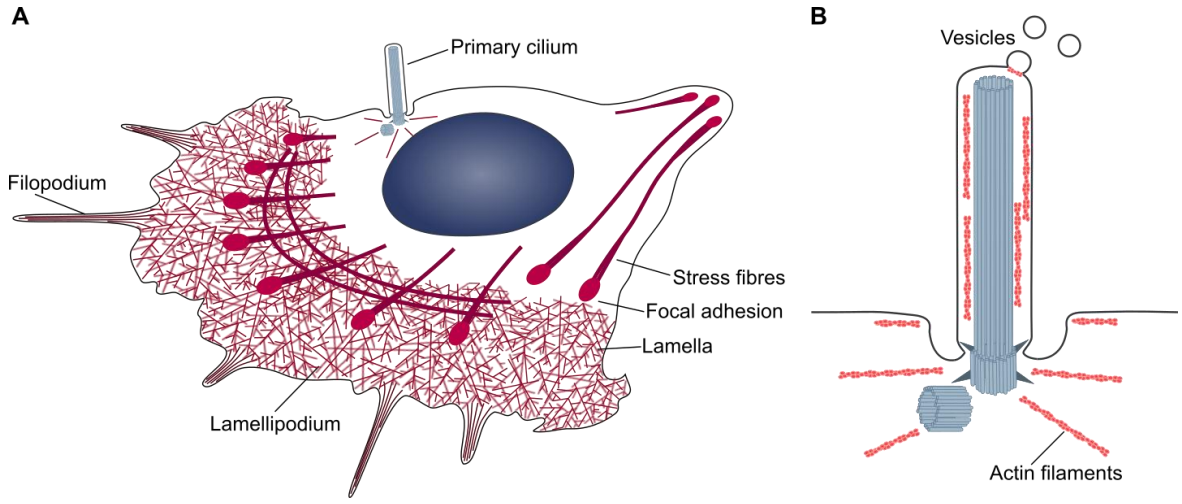

Fig. 2: The actin cytoskeleton.

A The main structures of the actin cytoskeleton include stress fibres that are terminated within focal adhesions, the lamella at the leading edge, which makes up the lamellipodium, and microspikes called filopodia that sense the surrounding environment. Filopodia contain parallelised F-actin structures, whereas the contractile stress fibres are made of anti-parallel fibres. The lamella is made of dense, branched actin networks. Besides these prominent structures, actin mesh is found throughout the whole cell body. Modified after (Letort et al., 2015). **B** Actin is found at the basal body, inside the ciliary axoneme and at the ciliary tip, where it is thought to facilitate the ectocytosis of vesicles as a way to disassemble primary cilia.

There is lots of evidence for a functional role of numerous actin proteins during early ciliary development. A stable actin network including actin motor proteins is required for transport of preciliary vesicles to the basal body (Hong et al., 2015; Wu et al., 2018). During preciliary vesicle transport and docking, the microtubule and actin crosslinking factor MACF1 is further required, emphasising the importance of both stable actin and microtubule networks during the initiation of ciliogenesis (May-Simera et al., 2016). Centrosome positioning and docking is also dependent on interactions between cilia proteins and the actin scaffolding protein Nesprin-2 that activates the RhoA-dependent apical actin network at the basal body (Dawe et al., 2009; Pan et al., 2007). At the basal body, the actin nucleators Cobl, Arp2/3 and its activator Wash, a member of the Wiskott Aldrich Syndrome protein and scar homologue complex family, are

recruited to induce actin filament nucleation, which is critical for centrosome positioning (Farina et al., 2016; Haag et al., 2018; Obino et al., 2016). The basal body is further connected to the actin network via focal adhesion proteins such as focal adhesion kinase (FAK), paxillin and vinculin (Antoniades et al., 2014). Thus, the basal body was suggested to be both a microtubule- and actin-organising centre (Farina et al., 2016). The subsequent formation of the ciliary pocket requires membrane tubulation which also involves the actin cytoskeleton (Saito et al., 2017).

Besides its role during early ciliogenesis, F-actin and actin regulators are involved in various aspects of cilia maintenance and disassembly. Despite the fact that the presence of F-actin is likely to be cell cycle and cell type-specific, F-actin was identified at the basal body (Farina et al., 2016; Molla-Herman et al., 2010; Saito et al., 2017), and in mature primary cilia inside the axoneme (Copeland et al., 2018; Kiesel et al., 2020; Lee et al., 2018; Phua et al., 2017) and even at the ciliary tip (Fig. 2B; Corral-serrano et al., 2020; Nager et al., 2017; Phua et al., 2017; Wang et al., 2019). Although the function of F-actin inside primary cilia is still not completely solved, it is thought to assist in the ectocytosis of ciliary vesicles as a way of ciliary disassembly (Corral-serrano et al., 2020; Nager et al., 2017; Phua et al., 2017; Spencer et al., 2019; Wang et al., 2019). Concomitantly, depolymerisation of F-actin was found to stabilise primary cilia (Bershteyn et al., 2010; J. Kim et al., 2010; Kim et al., 2015; Liang et al., 2016; Pitaval et al., 2010).

Taken together, the assembly, maintenance and disassembly of primary cilia requires both the microtubule and actin networks. Disturbance of these complex mechanisms affect cilia development and function. Since the correct function of primary cilia is crucial for tissue development and homeostasis, ciliary defects can cause a range of severe genetic disorders, collectively termed ciliopathies.

1.2. Ciliopathies

Ciliopathies arise due to defective cilia development, function or disassembly and comprise both organ-specific disorders and pleiotropic syndromes where several organs are affected. The total prevalence of ciliopathies in the population ranges from 1:700 to 1:2000 (Wheway et al., 2019). Prominent examples for syndromic ciliopathies are Meckel-Gruber syndrome, Joubert syndrome, Alström syndrome, McKusick-Kaufman syndrome and Bardet-Biedl syndrome, often showing symptomatic overlaps in terms of their phenotype. Since primary cilia can be found on almost all human cell types, the phenotypes associated with ciliopathies are wide-ranging and diverse. They include retinopathies, renal impairment, obesity, polydactyly, brain and skeletal abnormalities, mental retardation, and situs inversus (Chen et al., 2021; Hildebrandt et al., 2011; McConnachie et al., 2021). Besides these most commonly occurring primary symptoms, secondary features such as cardiovascular defects, respiratory abnormalities, hearing loss, genital impairments, and defects of the endocrine system can also arise (Focșa et al., 2021).

Table 1: Selected ciliopathies and overlapping syndromic features.

	BBS	**MKS**	**JBTS**	**JATD**	**OFD1**	**MKKS**	**SLS**	**NPHP**	**LCA**	**ALMS**
Retinopathy	✓	✓	✓	✓			✓	✓	✓	✓
Obesity	✓									✓
Polydactyly	✓	✓	✓	✓	✓	✓				
Kidney disease	✓	✓	✓	✓	✓		✓	✓		
Situs inversus	✓	✓	✓				✓	✓		
Cognitive impairment	✓	✓	✓		✓			✓	✓	✓

BBS: Bardet-Biedl syndrome; MKS: Meckel-Gruber syndrome; JBTS: Joubert syndrome; JATD: Jeune syndrome; OFD1: Oro-facial-digital syndrome type 1; MKKS: McKusick-Kaufman syndrome; SLS: Senior-Loken syndrome; NPHP: Nephronophthisis; LCA: Leber congenital amaurosis; ALMS: Alström syndrome

1.2.1. The Bardet-Biedl syndrome

Bardet-Biedl syndrome (BBS), one of the first ciliopathies to be described as early as 1866 and 1933 (Clay, 1933), is predominantly inherited in an autosomal recessive manner and characterised by high genetic heterogeneity. However, due to the absence of a genotype-to-phenotype-correlation, the clinical expressivity is highly variable and pleiotropic (Badano et al., 2006; Katsanis, 2004). As a flagship ciliopathy, the Bardet-Biedl syndrome combines many primary features such as rod-cone dystrophy, obesity, polydactyly, hypogonadism, kidney abnormalities and cognitive impairment (Beales et al., 1999; Florea et al., 2021; Forsythe and Beales, 2013).

1.2.1.1. BBS protein functions

Causative for the development of Bardet-Biedl syndrome are mutations in the *BBS* genes, encoding BBS proteins that execute critical ciliary functions. So far, 24 different BBS-related proteins have been identified that are listed below (Table 2; Florea et al., 2021). Some of these proteins are organised in complexes, such as the BBSome or the chaperonin-like complex which will be described in more detail below. Other BBS proteins such as BBS15-17 are involved in signalling pathways or in case of BBS19-20 the intraflagellar trafficking inside the cilium.

Table 2: BBS proteins, related functions and cellular localisation. Adapted from Florea et al., 2021.

BBS	Symbol	Protein Name	Function/Family	Localisation
1	BBS1	Bardet–Biedl syndrome protein 1	BBSome	Basal body, cilium
2	BBS2	Bardet–Biedl syndrome protein 2	BBSome	Basal body, cilium
3	BBS3/ARL6	ADP-ribosylation factor-like protein 6	ARF GTPase family BBSome recruitment	Basal body, cilium, cytosol, transition zone
4	BBS4	Bardet–Biedl syndrome protein 4	BBSome	Basal body, cilium
5	BBS5	Bardet–Biedl syndrome protein 5	BBSome	Basal body

6	BBS6/MKKS	McKusick–Kaufman syndrome protein	Chaperonin-like	Basal body, cytosol
7	BBS7	Bardet–Biedl syndrome protein 7	BBSome	Basal body, cilium
8	BBS8/TTC8	Tetratricopeptide repeat domain protein 8	BBSome	Basal body, cilium, IFT
9	BBS9	Bardet–Biedl syndrome protein 9	BBSome	Cilium
10	BBS10	Bardet–Biedl syndrome protein 10	Chaperonin-like	Basal body
11	TRIM32	Tripartite motif containing 32	E3 ubiquitin-protein ligase	Intermediate filaments
12	BBS12	Bardet–Biedl syndrome protein 12	Chaperonin-like	Basal body
13	MKS1	Meckel syndrome type 1 protein	B9 domain containing MKS complex	Basal body
14	CEP290	Centrosomal protein of 290 kDa	MKS complex	Basal body, centrosome
15	WDPCP	WD repeat containing planar cell polarity effector protein	Ciliogenesis and planar polarity effector complex	Cytosol, plasma membrane, axoneme
16	SDCCAG8	Serologically defined colon cancer antigen 8	MicroRNA protein coding, Shh signalling	Basal body, centriole, transition zone
17	LZTFL1	Leucine zipper transcription factor-like protein 1	BBSome antagonist, Wnt/Shh signalling	Basal body, cilium
18	BBIP1	BBSome-interacting protein 1	BBSome	Cytoplasm, cytosol
19	IFT27	Intraflagellar transport protein 27	IFT complex	Basal body, cilium, IFT
20	IFT74	Intraflagellar transport protein 74	IFT complex	Basal body, cilium, IFT
21	C8orf37	Chromosome 8 open reading frame 37 protein		Basal body, ciliary root

22	SCLT1	Sodium channel and clathrin linker 1		Centriole
23	NPHP1	Nephrocystin-1	NPHP complex	Transition zone
24	SCAPER	S-phase cyclin A associated protein in the ER	Zinc finger	ER

ARF: ADP-ribosylation factor; BBS: Bardet-Biedl syndrome; ER: Endoplasmic reticulum; IFT: Intraflagellar transport, MKKS: McKusick–Kaufman syndrome; MKS: Meckel syndrome; NPHP: Nephrocystins; Shh: Sonic hedgehog

The eight proteins BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9 and BBS18/BBIP1 are found in a complex, called the BBSome, that acts as an adaptor for proteins of the intraflagellar transport machinery, enabling cargo of proteins and vesicles into (anterograde) and out of (retrograde) the primary cilium (Blacque et al., 2004; Lechtreck et al., 2009; Nachury et al., 2007; Ye et al., 2018). In cooperation with the small Arf-like GTPase ARL6/BBS3 in its GTP-bound active state, the BBSome is recruited to cilia and regulates import and export of proteins into and out of the cilium (Fig. 3; Jin et al., 2010; Liew et al., 2014; Singh et al., 2020; Xue et al., 2020).

The assembly of the BBSome is facilitated by the BBS/CCT chaperonin complex consisting of the chaperonin-like BBS proteins BBS6, BBS10 and BBS12 and members of the CCT/TriC (Chaperonin containing TCP-1/T-complex protein-1 ring complex) family of chaperonins. The chaperonin-like BBS proteins BBS6, BBS10 and BBS12 share sequence homology with the chaperonin containing t-complex protein 1 of the CCT family of group II chaperonins (Kim et al., 2005; Stoetzel et al., 2007, 2006). Under hydrolysis of ATP, the CCT chaperonins are known to mediate the folding of cytoskeleton-associated proteins such as actin and tubulin and the assembly of the BBSome (Dunn et al., 2001; Sinha et al., 2014).

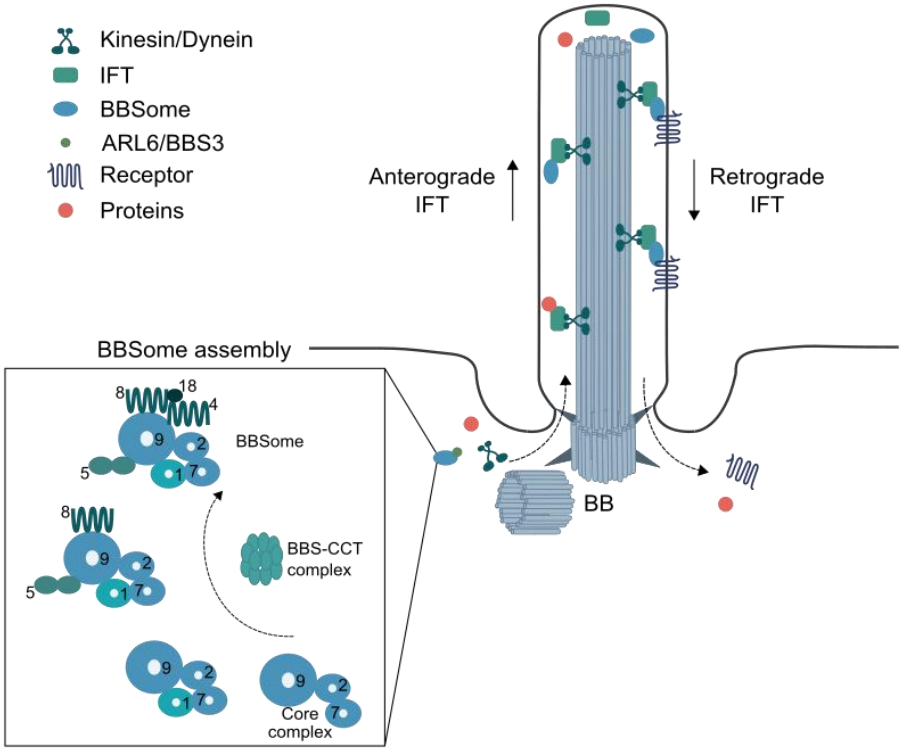

Fig. 3: BBSome assembly and IFT transport.

The BBSome (BBS1, 2, 4, 5, 7, 8, 9, 18) is assembled via a complex consisting of the chaperonin-like proteins BBS6, BBS10, BBS12 and CCT chaperonins. BBS2, BBS7 and BBS9 make up the BBSome core complex, whereas BBS4 is the last component to join (Chou et al., 2019). As soon as the BBSome is assembled, it is recruited to the cilium via ARL6/BBS3. The BBSome acts as an adaptor for IFT trains and regulates entry and export of proteins and signalling receptors required for cilia function. ARL6: ADP-ribosylation factor-like protein 6; BB: Basal body; CCT: Chaperonin containing TCP-1; IFT: Intraflagellar transport.

Since they share sequence homology with the CCT chaperonins, the protein structure of the three BBS proteins is simple but mostly evolutionary conserved, comprising of one apical domain that is flanked by intermediate and equatorial domains (Álvarez-Satta et al., 2017). The ATP hydrolysis motif in the equatorial domain is conserved in BBS10, but altered in both BBS6 and BBS12, which is why they are not thought to exert a folding function. Furthermore, due to several amino acid insertions, all BBS chaperonin-like proteins are not thought to be able to build a functional oligomeric complex like CCT chaperonins do (Kim et al., 2005; Mukherjee et al., 2010; Stoetzel et al., 2007, 2006). However, they play an important role in the assembly of the BBSome (Fig. 3). As a first step, BBS6 and BBS12 bind to and stabilise BBS7 and, in cooperation with BBS10, mediate its interaction with the CCT chaperonin complex (Seo et al., 2010; Zhang et al., 2012). The CCT complex, consisting of the CCT proteins CCT1-5 and

CCT8, further executes the folding of BBS7 (Sinha et al., 2014; Zhang et al., 2012). After release of BBS6 and BBS12 from the chaperonin-complex, BBS2 binds to BBS7, followed by BBS9, together generating the BBSome core complex. Subsequently, the CCT chaperonins are also liberated from the complex (Zhang et al., 2012). Due to intrinsic protein-protein-interactions, the remaining BBSome components join the complex, enabling its final recruitment to the primary cilium and its function in cargo trafficking. Structurally, BBS2 and BBS7 form the top portion of the BBSome, while BBS5, BBS8 and BBS9 form the base (Chou et al., 2019). BBS4 is the last BBSome component to be added as it moves from centriolar satellites to the BBSome shortly before cilia entry (Fig. 3; Chou et al., 2019; Loktev et al., 2008; Zhang et al., 2012).

Up to 50% of all diagnosed BBS patients carry pathogenic variants of the three chaperonin-like genes (Billingsley et al., 2010; Deveault et al., 2011; Muller et al., 2010). To be more precise, *BBS1* and *BBS10* are the most frequent mutated genes in individuals diagnosed with BBS, accounting together for more than 50% of the cases, followed by *BBS12* with 10% (Forsythe et al., 2015; Forsythe and Beales, 2013; Muller et al., 2010). It was also suggested that patients with mutations in the chaperonin-like *BBS* genes develop a more severe phenotype, including earlier onset of disease, higher prevalence of primary symptoms and more overlap with other ciliopathies such as Alström or McKusick-Kaufman syndrome (Billingsley et al., 2010; Castro-Sánchez et al., 2015; Imhoff et al., 2011). However, some mutations in the *BBS* genes, especially for recent identified genes such as *BBS21-BBS24*, were only identified in few individuals, where clinical features were crucial to identify them as *BBS* genes (Heon et al., 2016; Morisada et al., 2020; Wormser et al., 2019).

1.2.1.2. Actin-related BBS protein functions

Besides their well-described function in ciliogenesis, some ciliary proteins were found to affect various other aspects of cell homeostasis, such as cell cycle, transcriptional splicing, cytoskeleton organisation or cell migration (Lin et al., 2018; Wood et al., 2012; Yin et al., 2009). In particular BBS proteins have been highlighted in non-cilia related functions, such as intracellular trafficking (Leitch et al., 2014; May-Simera et al., 2015; Yen et al., 2006), cell cycle regulation (Delaval et al., 2011; Wood et al., 2012), proteasomal degradation (Gerdes et al., 2007; Kudryashova et al., 2005; Liu et al., 2014), DNA damage response (O'Regan et al., 2007), and transcriptional regulation (Gascue et al., 2012). Especially the regulation of BBS

proteins in actin networks is well documented (Gerdes et al., 2007; Hernandez-Hernandez et al., 2013; May-Simera, 2016; May-Simera et al., 2010; Ross et al., 2005; Tobin et al., 2008). More specifically, the BBSome components Bbs4 and Bbs8 were shown to be required for cell migration and division since they are involved in the regulation of filopodia and development of lamellipodia (Hernandez-Hernandez et al., 2013; Tobin et al., 2008). This was mainly due to organisation of the apical actin network and actin stress fibre polymerisation (Hernandez-Hernandez et al., 2013; May-Simera et al., 2010). In addition to their ciliary localisation, the BBSome proteins BBS8 and BBS9 were further found to localise to focal adhesions, suggesting a role of these proteins in the regulation of actin structures (Hernandez-Hernandez et al., 2013). Concordantly, Bbs4 and Bbs8 are thought to prevent focal adhesion formation by inhibiting actin polymerisation via regulation of RhoA signalling (Hernandez-Hernandez et al., 2013). Although BBS6 is not a component of the BBSome, but involved in its assembly via interaction with CCT chaperonins, a similar role for Bbs6 in the RhoA-dependent regulation of actin stress fibres has been postulated (Hernandez-Hernandez et al., 2013). Besides regulation of RhoA, BBS6 has been also shown to regulate the downstream actin and microtubule networks via interaction with the microtubule and actin crosslinking factor 1 (MACF1) (May-Simera et al., 2016, 2009).

The regulation of the ciliary BBS proteins in actin networks can be ascribed to their involvement in ciliary signalling which will be described below. Since Wnt signalling is one of the main ciliary signalling pathways that directly targets the downstream actin network, it is of particular interest in the present work.

1.3. Wnt signalling

Primary cilia are implicated in the regulation of several signalling pathways, such as Sonic hedgehog (Shh), platelet-derived growth factor (PDGF) or Wnt signalling (Bangs and Anderson, 2017; Lee, 2020; Umberger and Caspary, 2015). The Wnt signalling pathway is one of the main cilia-regulated pathways affecting downstream actin perturbations (Balmer et al., 2015; Corbit et al., 2008; Cui et al., 2013; Gerdes et al., 2007; May-Simera et al., 2015, 2010; McMurray et al., 2013; Wang et al., 2017).

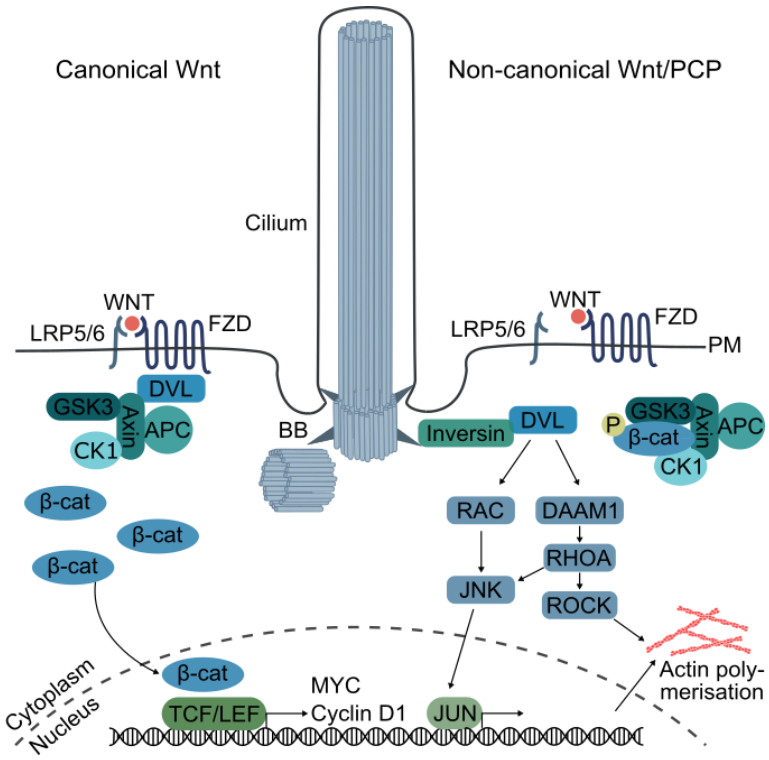

Fig. 4: The Wnt signalling pathway.

Graphical representation of canonical and non-canonical (PCP) Wnt signalling. During canonical Wnt signalling, the WNT ligand binds to a co-receptor complex consisting of LRP5/6 (low-density lipoprotein receptor-related proteins 5/6) and Frizzled (FZD). This results in activation of Dishevelled (DVL), which binds to and inactivates the β-catenin degradation complex consisting of APC (adenomatous polyposis coli), Axin, GSK3β (glycogensynthase kinase 3β) and CK1 (Casein kinase 1). β-catenin (β-cat) accumulates and enters the nucleus, where it activates the transcription of Wnt target genes such as Cyclin D1 or Myc. During PCP (planar cell polarity) signalling, binding of the WNT ligand results in the translocation of Dishevelled via Inversin, subsequently activating downstream signalling cascades involving RAC, DAAM1 (Dishevelled-associated activator of morphogenesis 1) and RHOA (Ras homolog family member A), leading to actin rearrangements. A slight variation of this figure is also included in Manuscript I. TCF: T-cell factor, LEF: lymphoid enhancer-binding factor, ROCK: Rho-associated, coiled-coil-containing protein kinase, JNK: c-Jun N-terminal kinase, JUN: c-Jun PM: plasma membrane, P: phosphorylation.

Wnt signalling is composed of two main branches: canonical or β-catenin dependent Wnt signalling and non-canonical Wnt, comprising the planar cell polarity (PCP) and Calcium dependent Wnt signalling pathways. Canonical Wnt signalling is controlled via precise regulation of cytosolic β-catenin levels (Fig. 4). During canonical Wnt, a coreceptor complex, the so-called signalosome, consisting of the G-protein coupled receptor Frizzled (FZD) and the low-density lipoprotein receptor-related proteins 5/6 (LRP5/6), is activated via binding of a WNT ligand (Bilić et al., 2007; Cong et al., 2004). WNT ligands can activate both canonical (β-catenin dependent) or non-canonical Wnt. Some of 19 total WNT ligands can preferentially affect one pathway, such as WNT3a which activates the canonical branch (Willert et al., 2003; Xu and Gotlieb, 2013) and WNT5a activating PCP signalling (Kikuchi et al., 2007). However, the specificity of WNT ligands in general is not completely set in stone, but rather receptor and cell-type specific (Kyun et al., 2020; Okamoto et al., 2014). Besides WNT ligands, other molecules can also target LRP receptors such as the Wnt antagonists Dickkopf-related protein 1 (DKK1) (Semënov et al., 2008, 2001), secreted Frizzled-related protein 1 (SFRP1), Wnt inhibitory factor (WIF), Sclerostin, and the Wnt agonists R-spondin and Norrin (Cruciat and Niehrs, 2013).

Binding of the Wnt ligand to the LRP5/6 signalosome results in the recruitment of Dishevelled (DVL), casein kinase 1 α (CK1α), glycogensynthase kinase 3β (GSK3β) and Axin to the receptor complex (Cong et al., 2004; Davidson et al., 2005; Krasnow et al., 1995), which leads to disassembly of the so-called β-catenin destruction complex (Cselenyi et al., 2008; Piao et al., 2008; Stamos et al., 2014). This complex, which is required for phosphorylation of β-catenin in the absence of WNT ligands, consists of Axin, adenomatous polyposis coli (APC), GSK3β and CK1α (Kishida et al., 1998; Rubinfeld et al., 1996). Due to its inhibition, unphosphorylated, active β-catenin can accumulate in the cytoplasm and is shuttled to the nucleus where it acts as a coactivator of the transcription factors T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) which initiate the transcription of Wnt target genes in a context-dependent manner (Behrens et al., 1996; Molenaar et al., 1996; Van de Wetering et al., 1997). This process is initiated by β-catenin detaching the co-repressor Groucho/transducin-like enhancer of split from TCF/LEF (Cavallo et al., 1998; Flack et al., 2017; Roose et al., 1998). Besides TCF/LEF, β-catenin was shown to bind to and recruit several coactivators such as B-cell lymphoma 9 (BCL9), BCL9-like (BCL9L), Pygopus (Pygo 1 or Pygo 2), or CREB-binding protein (CBP)/p300 in a context-dependent fashion (Hoffmans and Basler, 2007; Sustmann et al.,

2008). Subsequently, the activation of canonical Wnt signalling results in cell proliferation and differentiation.

During the planar cell polarity pathway (PCP), a non-canonical Wnt signalling branch, the Frizzled receptor is also activated via WNT ligands (Minegishi et al., 2017). This results in the recruitment of Dishevelled to the plasma membrane via the ciliary protein Inversin, where it interacts with actin regulators such as the small GTPase RAC1 or the formin DAAM1 (Habas et al., 2001; Simons et al., 2005). Consequently, signalling cascades are activated via actin regulators RhoA, ROCK and JNK, that lead to the transcription of cytoskeletal genes such as JUN, subsequently affecting subapical actin rearrangements (Habas et al., 2001; Liu et al., 2008).

During PCP signalling, β-catenin is phosphorylated via the destruction complex (Fig. 4). APC and Axin facilitate the CK1α dependent phosphorylation of β-catenin at residue Ser45 which then enables the phosphorylation via GSK3β at Ser33, Ser37 and Thr41 (Amit et al., 2002; Ikeda et al., 1998; Liu et al., 2002; Wu and He, 2006). Phosphorylated β-catenin is recognised by the Skp1-Cullin1-F-box (SCF) E3 ubiquitin ligase complex, which ubiquitinates β-catenin and induces its proteasomal degradation (Hart et al., 1999; Yanagawa et al., 2002; Yost et al., 1996). Besides being phosphorylated, β-catenin can also be acetylated at Lys49 via CBP regulating its transcriptional activity in a promoter specific fashion (Wolf et al., 2002). Since PCP signalling results in changes of the actin cytoskeleton, leading to the coordinated orientation of cells within a tissue, it is highly crucial for organ development (Gong et al., 2004; Luo et al., 2020).

1.3.1. Ciliary regulation of Wnt signalling

The balance between the Wnt signalling branches is highly crucial for cell homeostasis and during tissue development (Gong et al., 2004; Luo et al., 2020). In various tissues such as the retina or cochlea, disturbance of Wnt signalling was shown to result in defective tissue development leading to blindness or hearing loss (Kretschmer and May-Simera, 2020; Munnamalai and Fekete, 2013). Primary cilia regulate the switch between canonical and non-canonical Wnt signalling via the ciliary protein Inversin. In ciliated cells, Inversin is found at the basal body and transition zone of primary cilia, where it inhibits canonical Wnt during ciliogenesis, thus ciliary development occurs during non-canonical Wnt (Lienkamp et al., 2012; Simons et al., 2005). Concordantly, β-catenin was found to be recruited to mature primary cilia,

preventing its nuclear activity (Ajima and Hamada, 2011). Furthermore, loss of proteins required for cilia development leads to hyperactivation of canonical Wnt (Ajima and Hamada, 2011; Corbit et al., 2008; Gerdes et al., 2007). As bona-fide cilia proteins, BBS proteins were also found to directly influence ciliary Wnt signalling, consequently regulating downstream actin networks.

1.3.2. BBS proteins in Wnt signalling

In vivo data from various animal models suggest that the BBS proteins BBS4, BBS6 and BBS8 might be involved in non-canonical Wnt (PCP) signalling, subsequently affecting actin networks (Gerdes et al., 2007; May-Simera et al., 2010; Ross et al., 2005; Tobin et al., 2008). In zebrafish, knockout of *bbs6* and *bbs8* resulted in enhanced canonical Wnt signalling which consequently disrupts curvature of the body axis, a common readout for defective PCP (Gerdes et al., 2007; May-Simera et al., 2015, 2010). In mice, Bbs4 and Bbs6 were found to affect the polarisation of stereocilia hair bundles in the cochlea, another downstream effect of PCP signalling (Ross et al., 2005). Concordantly, BBS8 has been shown to interact with Inversin, key regulator of the switch between the Wnt branches (May-Simera et al., 2018), and with Vangl2, prominent PCP effector (May-Simera et al., 2015, 2010). Another example of BBS proteins being involved in the regulation of actin networks via PCP signalling is BBS15, also referred to as WDPCP, which is a well-known effector of non-canonical Wnt signalling (S. K. Kim et al., 2010). BBS15 further localises to and modulates actin stress fibres via interaction with the transition zone protein septin 2, subsequently enabling cell polarity, focal adhesion formation and cell motility (Cui et al., 2013). Taken together, these data show that the ciliary BBS proteins are involved in the regulation of Wnt signalling pathways, which consequently affects the downstream actin network. However, the precise regulations remain to be elucidated.

1.4. Objectives

To better understand of the molecular background underlying human ciliopathies, it is essential to know the exact function of ciliary proteins. This is not only important for understanding the disease and its complex phenotypic features but also to provide background for the development of potential therapeutics. In order to expand our knowledge of the function of ciliary proteins, this work aims to identify both ciliary and non-ciliary functions of the bona-fide BBS proteins. Bardet-Biedl syndrome as a model will help to approach this objective since BBS combines many clinical features, providing background to understand the molecular mechanisms of other ciliopathies as well. To reach this aim, three different yet overlapping objectives are formulated here: the expression levels of *Bbs* genes in different tissues will be first analysed to infer potential tissue-dependent functions of BBS proteins. Dependent on previous studies, the function of BBS proteins in Wnt signalling during tissue development will be examined. Subsequently, the downstream evaluation of ciliary proteins in actin dynamics and the role of actin proteins in ciliogenesis is of further interest to provide a broader background to contextualise the interplay between ciliogenesis, Wnt signalling and actin networks.

Objective 1: Investigation of potential tissue-dependent regulations of BBS proteins.

Since the ciliopathy Bardet-Biedl syndrome exhibits diverse phenotypes affecting many different organs of the human body, the first objective of the current work was to identify differences in gene expression of *Bbs* genes in different mouse tissues. The phenotypic occurrence of Bardet-Biedl syndrome is quite variable between patients, affecting various organs such as the retina, kidney, or gonads (Beales et al., 1999; Florea et al., 2021; Forsythe and Beales, 2013). This raises the possibility for tissue-dependent functions of BBS proteins and also potential non-cilia related functions. Thus, **Publication I** aims to get a better understanding of BBS protein functions within different tissues via analysis of comparative regulation of gene expressions.

Objective 2: Analysis of BBS functions in cilia-related Wnt signalling during RPE development.

The second objective aims to identify the role of BBS proteins during development of the retinal pigment epithelium (RPE), a ciliated monolayer in the eye crucial for visual function. Since BBS proteins were shown to be involved in Wnt signalling (Gerdes et al., 2007; May-Simera, 2016; May-Simera et al., 2015, 2010; Ross et al., 2005), the role of BBS proteins during this pathway in relation to ciliary disassembly was investigated in more detail in **Publication II**. Since Wnt signalling is a major pathway required for RPE development, this improves the understanding of the molecular pathways underlying the visual phenotype occurring in Bardet-Biedl syndrome and further elucidates the role for BBS proteins in Wnt signalling and its downstream effects.

Objective 3: Investigation into the connection between BBS, Wnt signalling and the downstream actin network.

Since BBS proteins are involved in Wnt signalling, which is tightly linked with a coordination of the downstream actin networks (Hernandez-Hernandez et al., 2013; Tobin et al., 2008), the interplay between BBS proteins and the actin network was investigated in **Manuscript I**. On the other hand, the actin network also plays an important role during ciliogenesis (Bershteyn et al., 2010; J. Kim et al., 2010; Kim et al., 2015; Liang et al., 2016; Pitaval et al., 2010), indicating potential feedback mechanisms between actin and cilia phenotypes. Thus, the complex regulations between actin and primary cilia were enlightened by reviewing the current literature in **Publication III**.

2. Publications and Manuscripts

Publication I

Patnaik SR, Farag A, Volz AK, **Brücker L**, Schneider S, Kretschmer V, May-Simera HL (2020): Tissue dependent differences in Bardet-Biedl Syndrome gene expression. *Biol Cell*; 112(2):39-52

Publication II

Patnaik SR, Kretschmer V, **Brücker L**, Schneider S, Volz AK, Oancea-Castillo LDR, May-Simera HL (2019): Bardet-Biedl Syndrome proteins regulate cilia disassembly during tissue maturation. *Cell Mol Life Sci*; 76(4):757-775

Manuscript I

Brücker L, Becker SK, Harms G, Parsons M, May-Simera HL: The actin-bundling protein Fascin-1 modulates ciliary signalling. Submitted at *JMCB* Feb 2022, in revision.

Publication III

Brücker L, Kretschmer V, May-Simera HL (2020): The entangled relationship between cilia and actin. Review. *IJBCB*; 129: 105877

Publication I

Biol. Cell (2020) **112,** 39–52 DOI: 10.1111/boc.201900077

Research article

Tissue-dependent differences in Bardet–Biedl syndrome gene expression

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Background Information. Primary cilia are highly conserved multifunctional cell organelles that extend from the cell membrane. A range of genetic disorders, collectively termed ciliopathies, is attributed to primary cilia dysfunction. The archetypical ciliopathy is the Bardet–Biedl syndrome (BBS), patients of which display virtually all symptoms associated with dysfunctional cilia. The primary cilium acts as a sensory organelle transmitting intra- and extracellular signals thereby transducing various signalling pathways facilitated by the BBS proteins. Growing evidence suggests that cilia proteins also have alternative functions in ciliary independent mechanisms, which might be contributing to disease etiology.

Results. In an attempt to gain more insight into possible differences in organ specific roles, we examined whether relative gene expression for individual *Bbs* genes was constant across different tissues in mouse, in order to distinguish possible differences in organ specific roles. All tested tissues show differentially expressed *Bbs* transcripts with some tissues showing a more similar stoichiometric composition of transcripts than others do. However, loss of *Bbs6* or *Bbs8* affects expression of other *Bbs* transcripts in a tissue-dependent way.

Conclusions and Significance. Our data support the hypothesis that in some organs, BBS proteins not only function in a complex but might also have alternative functions in a ciliary independent context. This significantly alters our understanding of disease pathogenesis and development of possible treatment strategies.

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Primary cilia are highly conserved multifunctional cell organelles that extend from the cell membrane. These microtubule-based appendages are vital for development and homeostasis of different organs and tissues and play a role in transduction of intra- and extracellular signals. In contrast to motile cilia, which are found on specialised tissues, primary cilia are a component of virtually all vertebrate cells, and functional defects cause a wide spectrum of clinical phenotypes. A range of genetic disorders, collectively termed ciliopathies, is attributed to primary cilia dysfunction. The archetypical ciliopathy is the Bardet–Biedl syndrome (BBS), patients of which display retinopathy, kidney dysfunction, obesity, polydactyly, behavioural dysfunction and hypogonadism [Forsythe and Beales, 2013].

The primary cilium acts as a sensory organelle transmitting intra- and extracellular signals [Ishikawa and Marshall, 2011] thereby transducing

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Key words: bardet-biedl syndrome, cilia, ciliopathy, gene regulation, mRNA.

Abbreviations: Arl6, ADP-ribosylation factor-like protein 6; ARVO, Association for Research in Vision and Ophthalmology; BBS, Bardet–Biedl syndrome; CCT/TRiC, chaperonin-containing TCP1 complex; cDNA, complementary deoxyribonucleic acid; DNA, deoxyribonucleic acid; DEPC, diethyl pyrocarbonate; GPCR, G protein-coupled receptor; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; IFT, intraflagellar transport; Mkks, McKusick-Kaufman syndrome; mRNAs, messenger ribonucleic acids; OD, optical density; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time polymerase chain reaction; RNA, ribonucleic acids; RNF2, RING finger protein 2; TOR, target of rapamycin; UK, United Kingdom; Usf1, upstream stimulatory factor 1.

various signalling pathways including Wnt [May-Simera and Kelley, 2012], Hedgehog [Goetz and Anderson, 2010], Notch [Ezratty et al., 2011], PDGF [Schneider et al., 2005], TOR [Yuan et al., 2013] and Hippo [Habbig et al., 2011]. Correct signal transduction is essential for tissue development and homeostasis and the Bardet–Biedl syndrome proteins have been shown to play a crucial role in this. To date, 23 BBS genes (BBS1 - 23) have been reported. Most recently two BBS disease causing loci have been found in other cilia associated genes, namely IFT74/BBS22 and SCAPER/BBS23 [Lindstrand et al., 2016; Schaefer et al., 2019; Wormser et al., 2019]. A subset of these genes encodes proteins that form an octameric complex termed the BBSome, which is crucial for ciliary trafficking [Jin et al., 2010]. The assembly of this complex is facilitated by chaperonin-like BBS proteins [BBS6/MKKS, BBS10 and BBS12; Seo et al., 2010; Zhang et al., 2012]. Although a defect in any BBS gene gives rise to a BBS diagnosis, there is a huge degree of phenotypic variation with no clear genotype to phenotype correlation even within families.

Growing evidence suggests that cilia proteins also have alternative functions in ciliary independent mechanisms. Such extraciliary functions include cell cycle regulation, non-ciliary trafficking, regulation of the DNA damage response, and transcriptional control [Vertii et al., 2015; Hua and Ferland, 2018]. In light of this, it is plausible that the BBSome and BBS chaperonin-like proteins also have alternative functions, possibly in an individual protein dependent manner. In an attempt to gain more insight into this, we examined whether relative gene expression for individual Bbs genes was constant across different tissues, in order to distinguish possible differences in organ specific roles. Furthermore, we wanted to examine the effect of Bbs protein loss on other BBSome or chaperonin-like components.

Results

Differential expression of BBSome transcripts across mouse tissues

To determine tissue specific expression levels of BBSome and chaperonin-like *Bbs* transcripts, we assessed their relative expression in a variety of adult mouse tissues. Quantitative real-time PCR (qRT-PCR) was used to examine the abundance of mRNA transcripts in brain, kidney, lung, spleen, heart, oviduct and retina. Because alternative splicing can produce transcripts with different stabilities, we designed primers to recognise as many of the different *Bbs* transcripts as possible (Table 1). An important consideration is that expression levels of housekeeping genes can vary across tissues, therefore caution must be used when comparing gene expression levels across multiple tissues and normalizing to expression of a single housekeeping gene. We chose *Gapdh* as a housekeeping control gene since the expression of *Gapdh* was more stable across the tissues examined than *Usf1* or other cytoskeletal markers (Supporting Information Figure 1). Furthermore we saw no differences in levels of *Gapdh* expression between either of our mutants and their littermate controls.

Our results show that *Bbs* transcripts are differentially expressed in different tissues when normalised against the housekeeping control *Gapdh* (Figure 1a–h). The expression levels of most BBSome transcripts were highest in the retina with the exception of *Bbs18* (Figure 1h). This correlates with the functional role of the BBSome in trafficking across the connecting cilium in photoreceptors [Datta et al., 2015]. Although the BBSome has a trafficking role in all cilia, the volume of traffic required to build and maintain the photoreceptor outer segment is particularly high and requires continuous turnover of ciliary trafficking proteins. Expression of *Bbs18* was most abundant in the spleen and oviduct.

Expression of BBSome transcripts within a tissue is not stoichiometric

Comparison of BBSome expression levels within a specific tissue revealed variable BBSome composition across different tissues. Expression of BBSome mRNAs were not stoichiometric, rather they were differentially expressed in specific tissues (Figure 2a–g). Interestingly, the expression profiles in brain and kidney were strikingly similar (Figures 2a and 2b). *Bbs1* was the most abundantly expressed transcript in brain and kidney, yet one of the least abundant transcripts in heart and oviduct (Figure 2a, 2b, 2e, and 2f). *Bbs18* was most abundant in the spleen, heart and oviduct, yet contributed the least in the retina (Figure 2g). Interestingly, *Bbs9* was highly abundant in the retina and spleen (Figures 2d and 2g). However, mutations in the *BBS9* gene have been implicated in nonsyndromic craniosynostosis [Barba et al., 2018]. These results

Table 1 | Gene symbol, primer sequences, primer efficiency for each evaluated Primer

Gene symbol	Primer sequencesForward n Reverse	Primer efficiency	Transcript identified
Bbs1	CCCTACTTCAAGTTCAGCCTG TCTGCCTTTTCCCTGATGTC	114.35%	ENSMUST00000053506.7
Bbs2	ACATTGCCCCACCTCTTG TCTTCCCATCACCGTCAAAG	115%	ENSMUST00000034206.5
Bbs3	GATACCCTTCTGAATCACCCAG CCACGGCTTGTCTTTAATGC	107.23%	ENSMUST00000023405.9 ENSMUST00000099646.3 ENSMUST00000118438.1 ENSMUST00000149797.1
Bbs4	GCTCCAGACTTCCCTATTGTG GCATATTCACATAGCCCCTGAG	109.34%	ENSMUST00000026265.7
Bbs5	ACAAAGTCTATTCTGCCAGTCC AAATACGCCACAAAAGCATCC	98.31%	ENSMUST00000074963.8 ENSMUST00000112286.8 ENSMUST00000134659.7
Bbs6	GTGTGCTCTGCAAGATTTGG AAGACGTGCATTGCTGTTTG	97.74%	ENSMUST00000110089.8 ENSMUST00000028730.12
Bbs7	ATGGATCTGACGTTAAGCCG CCTTTTGTGTAGCCCTTTGTCTTGAGGT	112.60%	ENSMUST00000108156.8 ENSMUST00000040148.10 ENSMUST00000108155.7 ENSMUST00000129671.1
Bbs8	GAGGCAGCTGATGTCTGGTACA CATTGGTGGGCCAAGTTTGT	98.25%	ENSMUST00000085109.9 ENSMUST00000079146.12
Bbs9	ACAAATCTCCTGTCAGTCTGC TCGTTGGGATGTTCTGGAAG	96.84%	ENSMUST00000147712.7 ENSMUST00000150395.7 ENSMUST00000039798.15 ENSMUST00000147405.7 ENSMUST00000127296.7
Bbs10	TCCAGCCTCAGTTTTCATCG ACTGAGATGCCTGAAACTGTG	111.39%	ENSMUST00000040454.4 ENSMUST00000219990.1
Bbs12	CGCCGAGCATTGGATGTAG CATGCACACCCACACGT	104.30%	ENSMUST00000057975.7 ENSMUST00000108121.3
Bbs18	CCCTTAAAATCTCTGACGCTGG TGCCTTTTCTGCCATTTCTTG	102.75%	ENSMUST00000135402.3 ENSMUST00000236885.1 ENSMUST00000235348.1 ENSMUST00000236370.1 ENSMUST00000236098.1 ENSMUST00000235688.1 ENSMUST00000237049.1
Gapdh	CGACTTCAACAGCAACTCCCACTCTTCC TGGGTGGTCCAGGGTTTCTTACTCCTT	99.58%	ENSMUST00000118875.7 ENSMUST00000117757.8 ENSMUST00000073605.14 ENSMUST00000183272.1

suggested either tissue-dependent differences in BBSome composition and/or protein half-life, or that some of these transcripts are required for alternative non-BBSome-related functions in different tissues.

Less variable expression of BBS chaperonin-like transcripts across mouse tissues

Next, we determined the gene expression patterns of the three chaperonin-like *Bbs* transcripts (*Bbs6/Mkks, Bbs10* and *Bbs12;* Figure 3). Overall, we saw less variation of these transcripts across different tissues. With exception of lung and heart, which both had slightly higher levels of expression; the level of *Bbs6* was relatively constant (Figure 3a). The expression pattern of *Bbs10* was largely consistent across different tissues (Figure 3b). *Bbs12* expression varied the most, with lower expression in brain and kidney and higher expression in the spleen, oviduct and retina (Figure 3c).

When looking at these expression levels within specific tissues, we observe a similar stoichiometry of expression in brain and kidney (Figure 3d,e), akin

Figure 1 | Expression of BBSome transcripts

Bar charts showing gene expression of *Bbs1,2,4,5,7,8,9,18* in different tissues relative to *Gapdh*. Relative expression levels of each sample averaged. Error bars show standard error of the mean. $n = 4$ for all genes, *Bbs8* $n = 3$. Statistics were done using the Dunnett's multiple comparison test $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{*}p \leq 0.001$; ns, not significant.

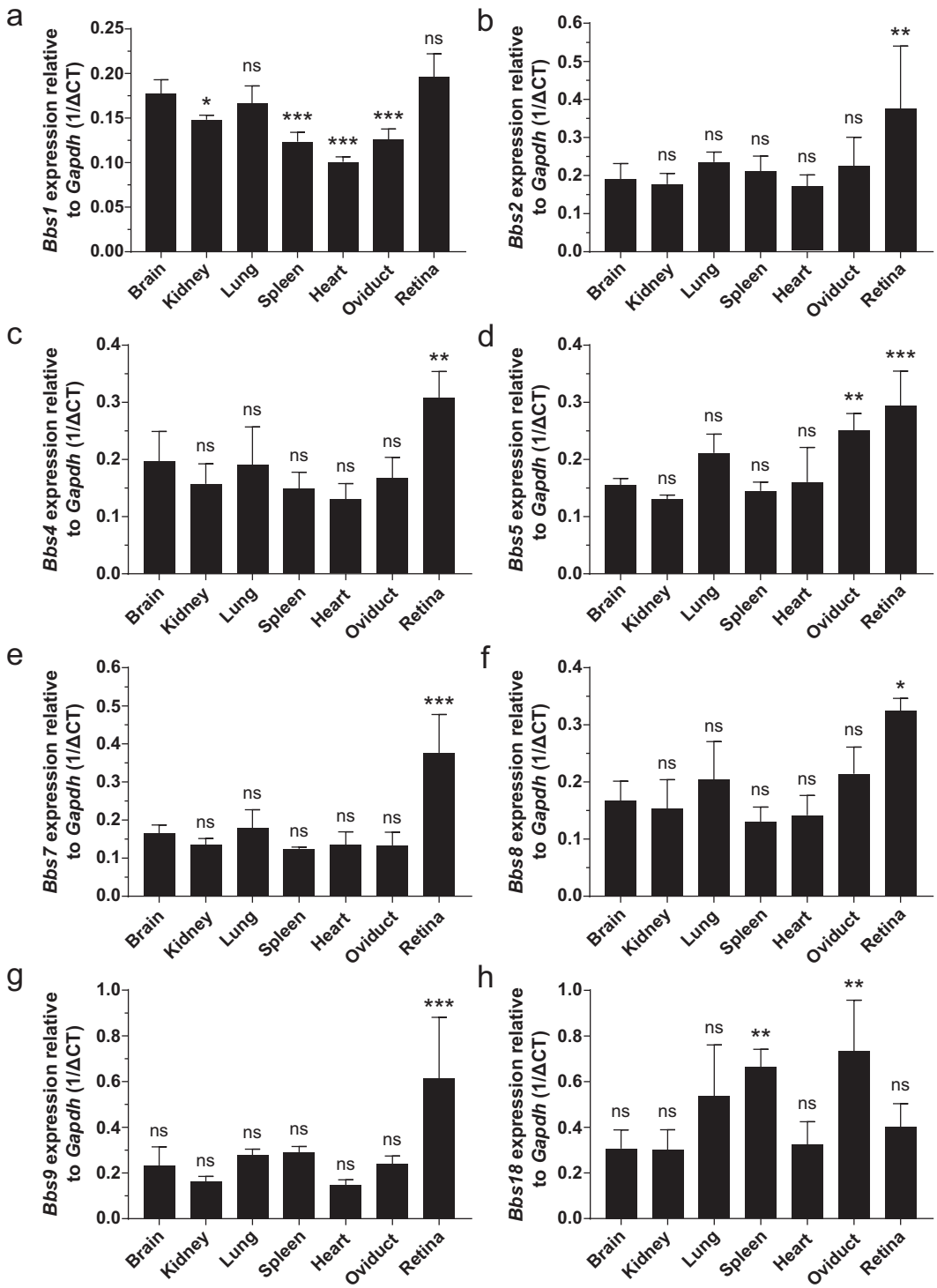
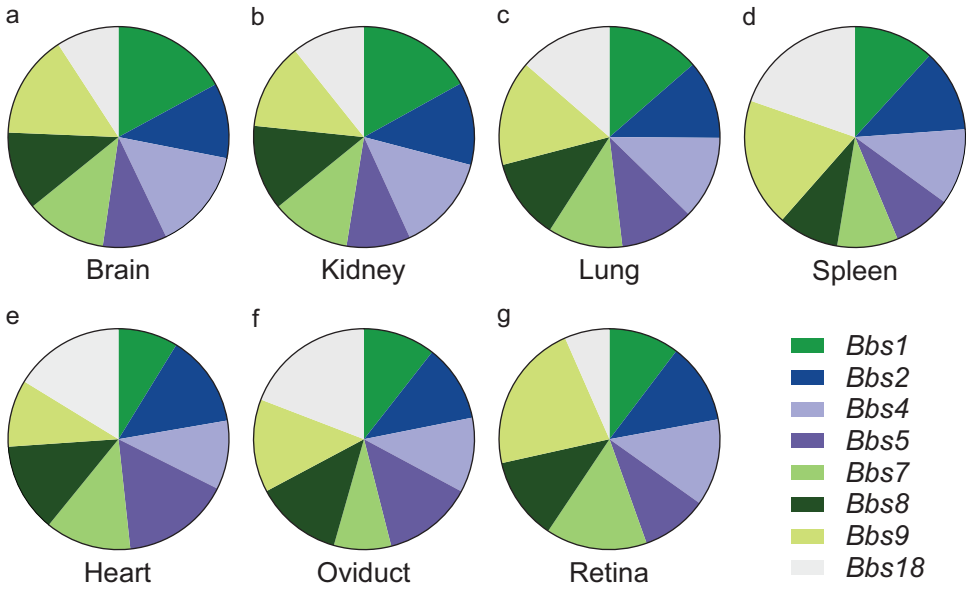


Figure 2 | Relative expression of BBSome transcripts

Pie charts showing relative gene expression of *Bbs1,2,4,5,7,8,9,18* in each tissue. Total expression of all BBSome transcripts is set at 100%. $n = 4$ for all genes, *Bbs8* $n = 3$.



to the trend seen for the BBSome transcripts. The one transcript that stands out is *Bbs12,* which is comparatively more abundant in spleen, oviduct and retina (Figure 3f–j). Overall, relative chaperonin-like *Bbs* expression levels within a specific tissue was less variable across tissues as compared to BBSome transcripts (Figures 3d–j and 2a–g).

Loss of Bbs8 leads to altered expression of other Bbs transcripts

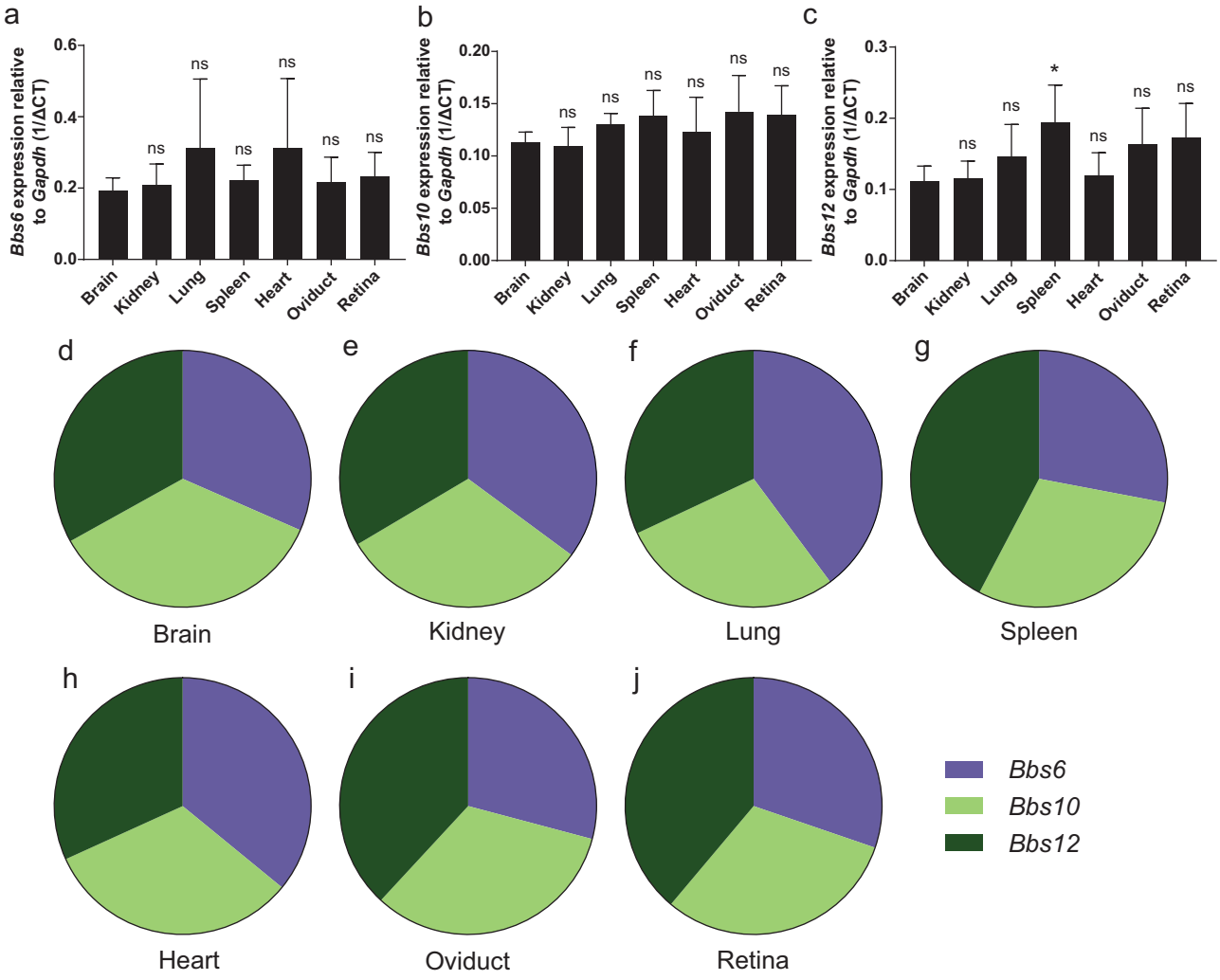
Previous studies from our lab have shown aberrant gene expression of ciliary proteins upon loss of BBS function [Patnaik et al., 2019]; however, very little is known about the transcriptional control of BBSome genes upon loss of one subunit. To assess the expression of BBSome subunits in the absence of *Bbs8/Ttc8*, we utilised a knock out mouse model. We measured mRNA expression levels of the other seven BBSome components as well as the three chaperonin-like genes in tissues harvested from adult $Bbs8^{+/+}$ and $Bbs8^{-/-}$ littermate mice.

We observed significant changes in BBSome mRNA levels in the absence of *Bbs8* suggesting a possible transcriptional control mechanism (Figure 4a–n). mRNA levels were either lower or unchanged in $Bbs8^{-/-}$ mice compared to $Bbs8^{+/+}$ in all tissues analysed (Figure 4a–n). We did not see a significant increase in mRNA expression of other BBSome subunits in an attempt to compensate for the loss. Intriguingly, the expression of *Bbs7* was significantly lower in all knock out tissues tested, while *Bbs9* did not show any significant changes in expression (Figures 4e and 4f). *Bbs18* was only downregulated in heart. Similar to what was observed above, expression patterns in brain and kidney were remarkably similar. In these two tissues, only *Bbs7* was significantly downregulated (Figures 4h and 4i). This could possibly suggest a similar mechanism of transcriptional control in these two organs. Other organs (lung, spleen, heart, oviduct, and retina) have numerous BBSome subunits that are significantly less expressed in $Bbs8^{-/-}$ mice compared to control (Figure 4j–n). Since *Bbs3* (*Arl6*) has extremely close functional links to the BBSome [Fan et al., 2004; Klink et al., 2017], we also analyzed the expression levels of this gene in $Bbs8^{-/-}$ tissues. We found that *Bbs3* had a unique pattern of expression change that did not resemble any of the changes in expression for individual BBSome components (Supporting Information Figure 2a).

We next assessed the mRNA expression levels of chaperonin-like components in $Bbs8^{+/+}$ and $Bbs8^{-/-}$ tissues (Figure 5a–j). Again, we observe tissue- and

Figure 3 | Expression of BBS chaperonin-like transcripts

a–c) Bar chart showing gene expression of *Bbs6, Bbs10* and *Bbs12* in different tissues relative to *Gapdh*. Relative expression levels of each sample averaged. Error bars show standard error of the mean. Statistics were done using the Dunnett's multiple comparison test *$p \leq 0.05$; ns not significant. **d–j**) Pie charts showing relative gene expression of *Bbs6/Mkks, Bbs10* and *Bbs12* in each tissue. Total expression of all Bbs-chaperonin transcripts is set at 100%. $n = 5$ for *Bbs10* and 12, *Bbs6* $n = 3$.



gene-dependent differences. Loss of *Bbs8* affected the expression levels of all three transcripts, *Bbs6/Mkks*, *Bbs10* and *Bbs12,* variably (Figure 5a–c). In mutant mice *Bbs6* expression was reduced compared to control in brain, oviduct and retina, while *Bbs10* showed lower expression in lung and heart (Figures 5a and 5b). The expression of *Bbs12* was comparable between mutant and control with the exception of oviduct, in which *Bbs12* was increased (Figure 5c). This was the only case in which loss of *Bbs8* lead to an increase in expression of an alternative *Bbs* gene. When focusing on individual tissues, brain and retina had a similar change in expression pattern, as well as lung and heart (Figures 5d, 5j, 5f and 5h). Decreased expression of *Bbs6* is only compensated by increased expression of *Bbs12* in oviduct (and not in brain or retina). This highlights a possible importance of *Bbs6* in the reproductive system. The expression of chaperonin-like genes were unaffected in kidney and spleen (Figures 5e and 5g).

Figure 4 | Expression of BBSome transcripts in *Bbs8*$^{-/-}$ tissues

a–g) Bar chart showing gene expression of *Bbs1,2,4,5,7,8,9,18* in different tissues from *Bbs8*$^{-/-}$ tissues relative to control *Bbs8*$^{+/+}$ (red line). Coloured bars indicate significantly downregulated genes. Relative expression levels of each sample averaged. Error bars show standard error of the mean. ***$p \leq 0.001$,**$p \leq 0.01$,*$p \leq 0.05$. **h–n**) Graphical representation of different tissue with respective genes downregulated (coloured boxes) in *Bbs8*$^{-/-}$ tissues relative to control. Experiments were performed in triplicates from three individual animals.

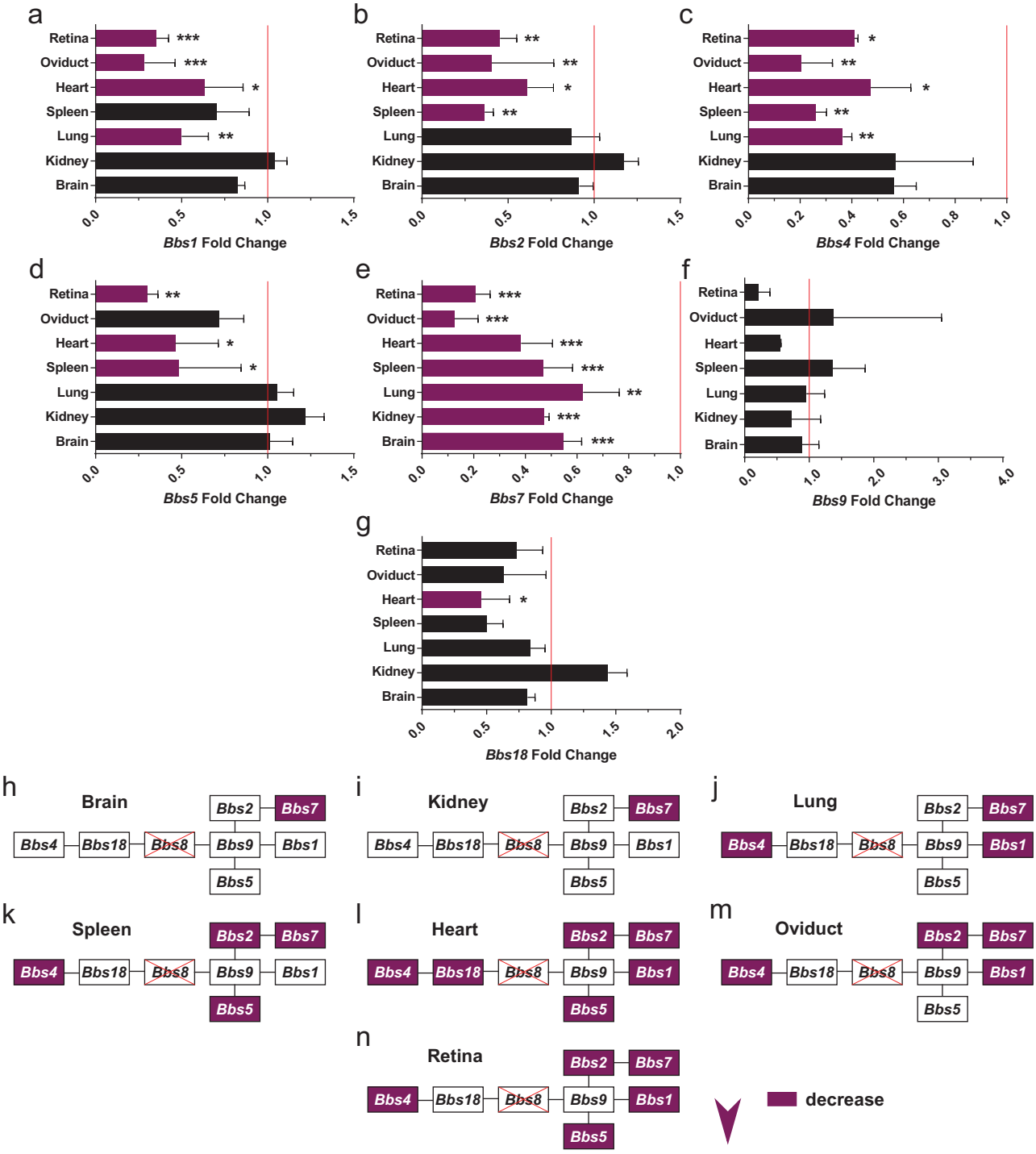
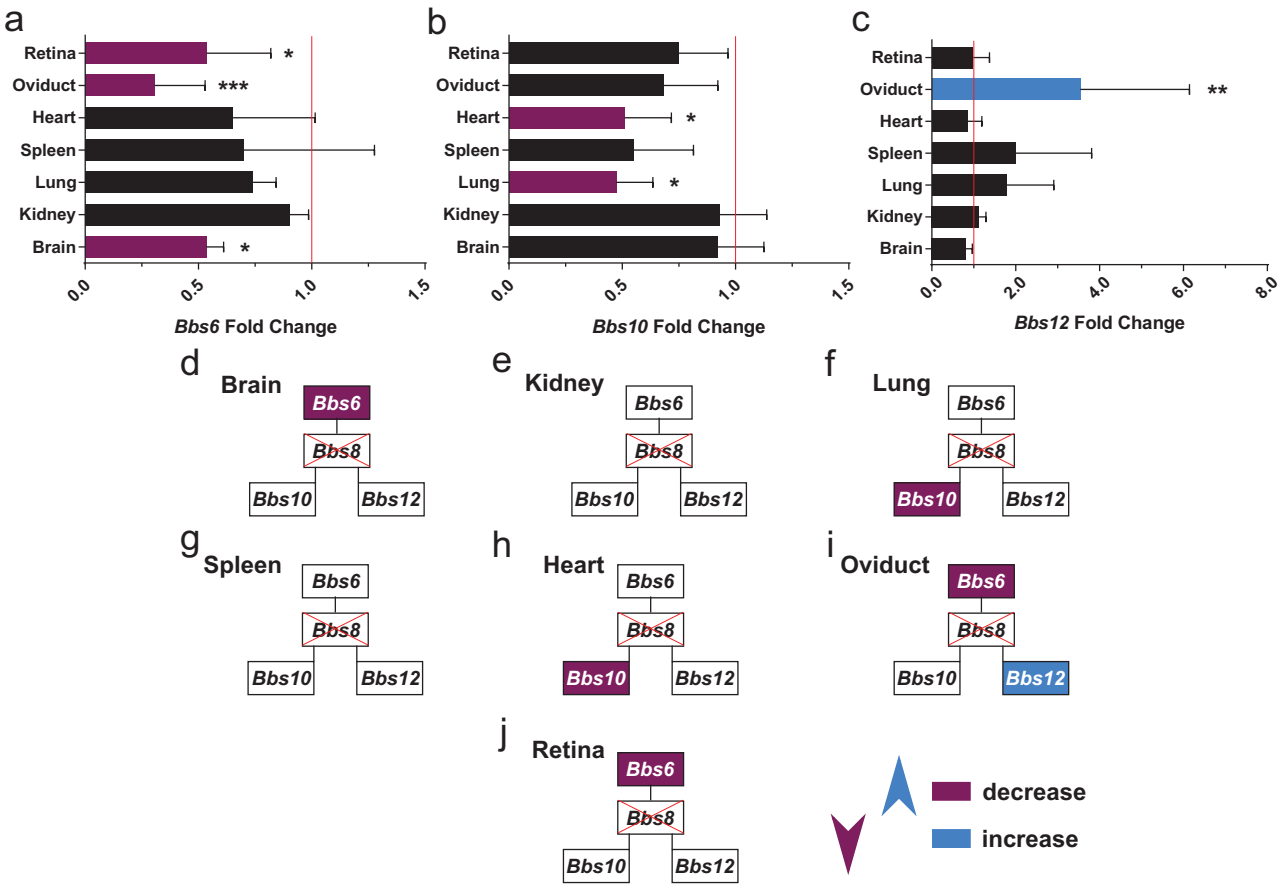


Figure 5 | Expression of BBS chaperonin-like transcripts in *Bbs8*$^{-/-}$ tissues

a–c) Bar chart showing gene expression of *Bbs6, 10 and 12* in different tissues from *Bbs8*$^{-/-}$ tissues relative to control *Bbs8*$^{+/+}$ (red line). Purple coloured bars indicate significantly downregulated genes, blue coloured bar indicates significantly upregulated gene. Relative expression levels of each sample averaged. Error bars show standard error of the mean. ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. **d–j**) Graphical representation of different tissues with respective genes downregulated or upregulated (coloured boxes) in *Bbs8*$^{-/-}$ tissues relative to control. Purple indicates significantly downregulated genes, blue significantly upregulated genes. Experiments were performed in triplicates from three individual animals.



Effect of Bbs6 loss on other Bbs transcripts

Lastly, we examined the loss of a BBS chaperonin-like component on *Bbs* transcript expression. We started by analysing the expression variability of BBSome components in different tissues in *Bbs6*$^{-/-}$ adult mice compared to *Bbs6*$^{+/+}$ littermate controls. Perhaps unsurprisingly, in contrast to *Bbs8*$^{-/-}$ mutant mice, the expression of BBSome mRNA remained stable in most tissues compared to the control, with the exception of spleen and lung (Figure 6a–o). All BBSome components except for *Bbs2* showed significantly higher expression in spleen (Figure 6l). In *Bbs6*$^{-/-}$ lung *Bbs2, Bbs4* and *Bbs18* expression was increased (Figure 6k). This increased expression (albeit only in spleen and lung) is in contrast to the decreased expression of BBSome transcripts in *Bbs8*$^{-/-}$ mice. As for the *Bbs8*$^{-/-}$ tissue, we also analyzed the expression levels of *Bbs3* in *Bbs6*$^{-/-}$ tissues. Similarly, we found that *Bbs3* also had a unique pattern of expression change upon loss of *Bbs6* (Supporting Information Figure 2b).

Similar to the BBSome transcripts, expression levels of chaperonin-like components were also less variable in *Bbs6*$^{-/-}$ adult mice compared to controls (Figure 7a–i). The only differences were observed in spleen, in which *Bbs10* was significantly increased

Figure 6 | Expression of BBSome transcripts in *Bbs6*$^{-/-}$ tissues

a–h) Bar chart showing gene expression of *Bbs1,2,4,5,7,8,9,18* in different tissues from *Bbs6*$^{-/-}$ tissues relative to control (red line). Coloured bars indicate significantly upregulated genes. Relative expression levels of each sample averaged. Error bars show standard error of the mean. ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$. **i–o**) Graphical representation of different tissues with respective genes upregulated (coloured boxes) in *Bbs6*$^{-/-}$ tissues relative to control. Experiments were performed in triplicates from three individual animals.

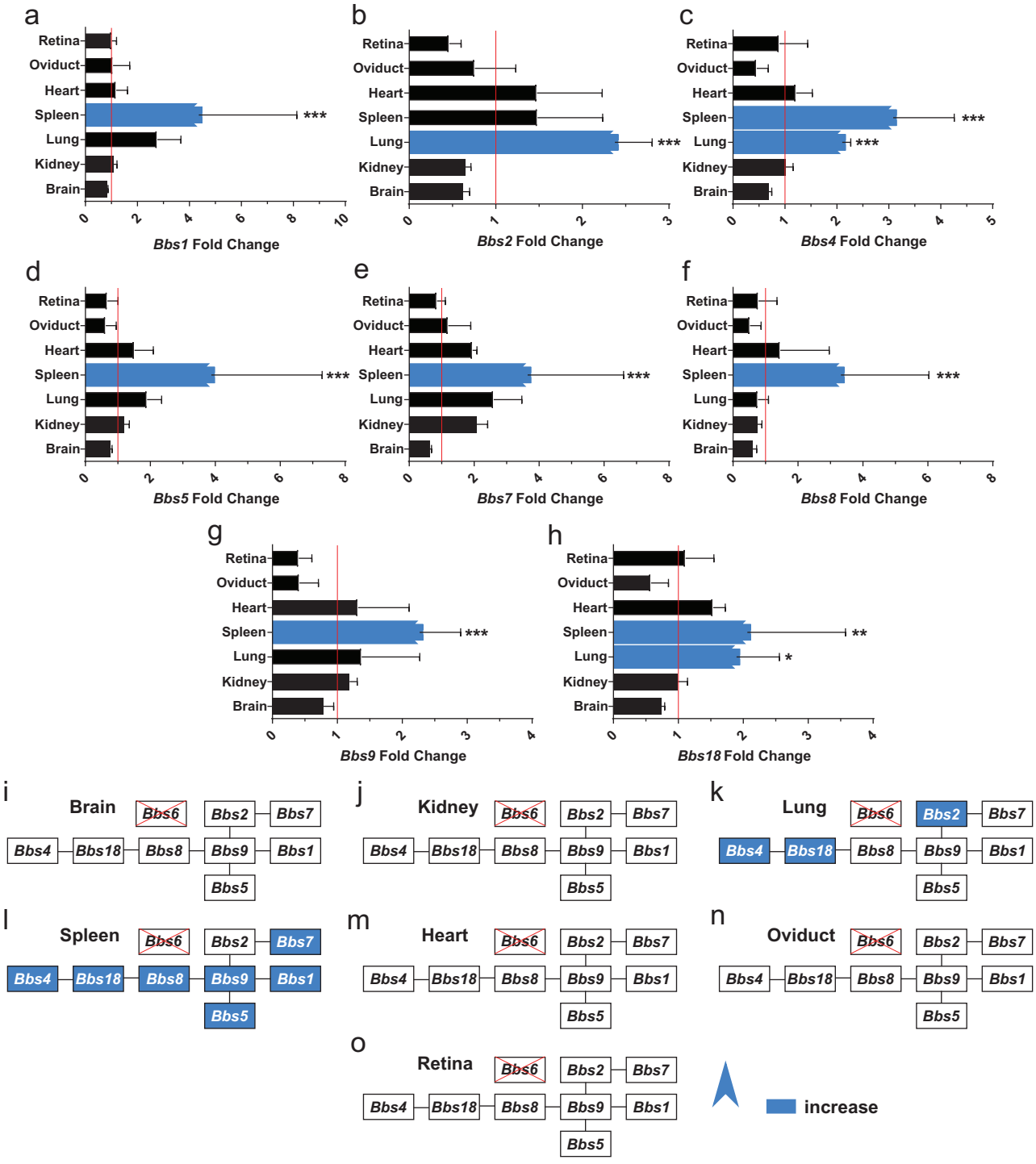
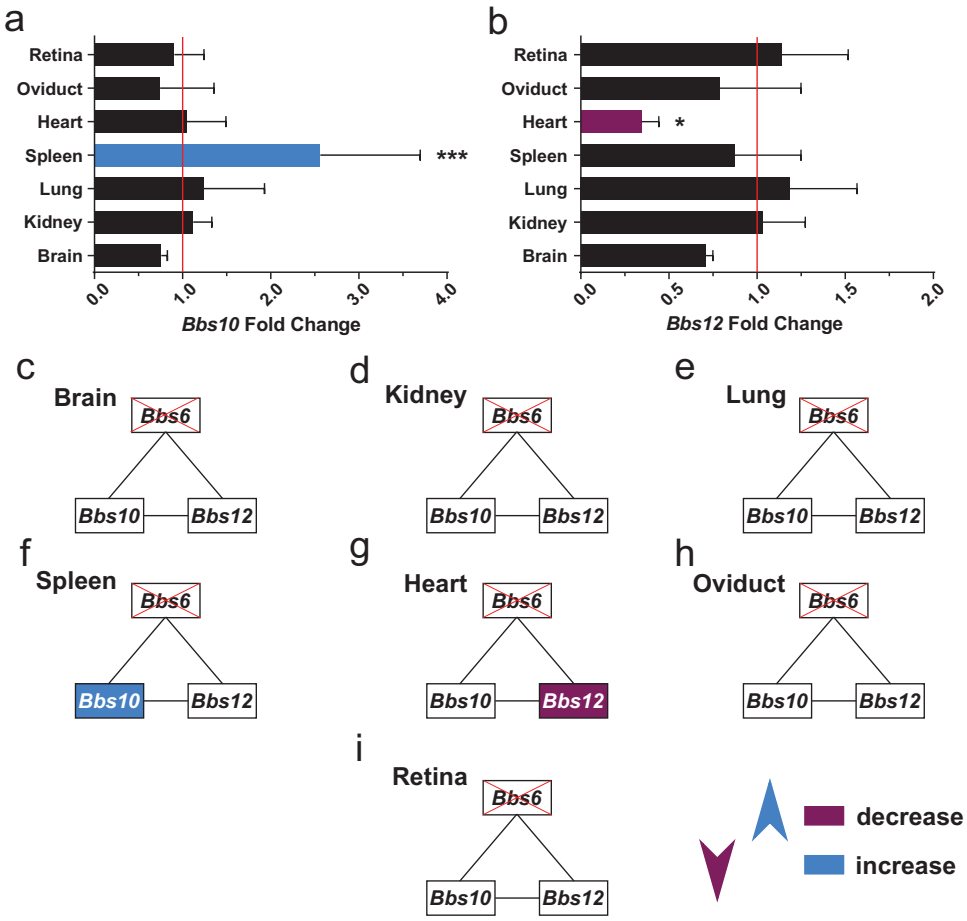


Figure 7 | Expression of BBS chaperonin-like transcripts in *Bbs6*$^{-/-}$ tissues

a and **b**) Bar chart showing gene expression of *Bbs6, 10 and 12* in different tissues from *Bbs6*$^{-/-}$ tissues relative to control (red line). Blue coloured bar indicates significantly upregulated gene, purple coloured bars indicates significantly downregulated gene. Relative expression levels of each sample averaged. Error bars show standard error of the mean. ***$p \leq 0.001$, **$p \leq 0.01$,*$p \leq 0.05$. c–i) Graphical representation of different tissues with respective genes upregulated or downregulated (coloured boxes) in *Bbs6*$^{-/-}$ tissues relative to control. Blue indicates significantly upregulated genes, purple significantly downregulated genes. Experiments were performed in triplicates from three individual animals.



(Figure 7a and 7f), and in heart in which *Bbs12* was significantly decreased (Figures 7b and 7g). Overall, loss of the chaperonin-like component *Bbs6* did not have a profound effect on transcripts encoding BBSome or chaperonin-like mRNA expression.

Discussion

Mutations in *BBS* genes cause a multitude of phenotypes affecting various organs and tissues. The predominant understanding is that BBS proteins facilitate ciliary membrane trafficking. In this context, the BBSome proteins (BBS1, 2, 4, 5, 7, 8, 9 and 18) form an octomeric protein complex that bind GPCRs and other receptors as a cargo adaptor during IFT [Nachury et al., 2007; Klink et al., 2017; Liu and Lechtreck, 2018]. In particular, BBSome-mediated trafficking is crucial for retrieval of GPCRs back into the cell [Wei et al., 2012; Nager et al., 2017; Ye et al., 2018]. The *BBS* chaperonin-like genes encode proteins (BBS6, 10, 12) that form a complex with the CCT/TRiC family chaperonins which is essential for BBSome assembly. However, the idea that BBS proteins are only functional in a ciliary context

might be an oversimplification. In recent years, there has been increasing interest in highlighting functions of ciliary proteins at extra-ciliary sites and in non-ciliary contexts. Such functions include intracellular trafficking, regulation of the cytoskeleton, mitosis, cell cycle regulation, regulation of the DNA damage response and transcriptional control [Vertii et al., 2015; Hua and Ferland, 2018]. Several studies also suggest that some of the BBS proteins might take on extra-ciliary roles, possibly in a non BBSome/chaperonin dependent manner that could be relevant to the aetiology of the disorder [Novas et al., 2015]. Alternative functions might be dependent on both time point and tissue type.

To gain more insight into BBS functionality, we set out to examine whether relative gene expression for individual *BBS* genes was constant across different tissues. We wanted to test the stoichiometric composition of *Bbs* at the level of mRNA across tissues. We postulated that if the BBSome and Bbs chaperonin-like genes only encode proteins that function in a defined complex, then their relative expression levels should be stoichiometrically conserved between different tissues.

We found that *Bbs* transcripts are differentially expressed in different tissues. Overall, there was a higher degree of variation among BBSome transcripts as opposed to BBS chaperonin-like transcripts. These results suggested possible tissue-dependent differences in BBSome composition and/or protein half-life, or that some of these transcripts might also be required for alternative non-BBSome-related functions in different tissues. A more stoichiometric expression of BBS chaperonin-like transcripts suggests that their individual functions might be more closely coupled. Interestingly, several tissues had similar distribution of expression, which might allude to similar functionality on a molecular level. For example in brain and in kidney, the stoichiometry of both BBSome and BBS chaperonin-like transcripts were more similar compared to other tissues.

When we analysed mRNA expression of other BBSome components upon loss of BBSome component Bbs8, we observed that numerous transcripts were decreased, which might suggest a transcriptional control mechanism. Alternatively loss of Bbs8 might have a stronger impact on tissue health or function and therefore a greater impact on overall gene transcription. Surprisingly, we did not see a significant increase in mRNA expression of other BBSome subunits that would suggest compensation for the loss of *Bbs8*. Interestingly there was little consistency in which transcripts were affected with the exception of *Bbs7,* which was significantly decreased in all tissues examined, and *Bbs9,* which was stable across tissues. Loss of *Bbs8* also affected the expression of individual chaperonin-like genes differently. *Bbs6* and *Bbs10* were decreased in several tissues. In contrast, *Bbs12* was found to be upregulated in oviduct. As in the control tissues, several tissues "responded" in a similar manner, such as brain and kidney in terms of BBSome transcripts and brain and retina in terms of chaperonin-like transcripts, alluding to similar functionalities of these molecules in these tissues.

Loss of BBS chaperonin-like component Bbs6 had little effect on the expression of BBSome transcripts in most tissues, yet surprisingly virtually all BBSome mRNAs in spleen and half in lung were upregulated. This might suggest that Bbs6 has some alternative role in spleen and lung tissue that is not present in any of the other tissues. Loss of *Bbs6* also increased the expression of the other chaperonin-like component, *Bbs10,* but only in spleen. Again, this suggests a possible alternative molecular function of Bbs6, which is possibly more prevalent in spleen. Although spleen defects are not readily reported in BBS patients.

Since alternative splicing can produce transcripts with different stabilities, we designed primers to recognise as many of the different BBS transcripts as possible. Nonetheless, alternative transcripts could result in different mRNA levels, which might contribute to the differences in expression levels described above. Absolute expression values must always be measured in relation to a housekeeping control the selection of which is crucial. We chose *Gapdh*, since it was the best option available to us, but are aware that variation in expression of housekeeping controls across tissues, which can always distort comparisons.

While our approach reveals differences in transcription that presumably affect protein abundance and consequent function, we were unable to show this directly due to technical limitations related to BBS proteins. Antibodies against these proteins are notoriously inconsistent and difficult to use. Numerous Western blots were performed using various tissues but give that certain antibodies failed or either detected bands at incorrect sizes or in knock

out tissues, the data were unreliable. To overcome issues related to antibody specificity, protein abundance was also quantified via mass spectroscopy but proved imprecise due to the relatively low abundance of Bbs proteins in each sample.

As mentioned above previous studies have already shown that BBS protein functions are not restricted to the primary cilium. Such functions include intracellular trafficking. BBSome components have been found to assist retrograde dynein mediated melanosome transport in zebrafish [Yen et al., 2006] as well as trafficking of various receptor molecules (Notch and Vangl2) to the cell membrane [Leitch et al., 2014; May-Simera et al., 2015]. An active role in regulation of the cytoskeleton has also been shown for BBS4, 6 and 8 via manipulation of actin polymerisation [Hernandez-Hernandez et al., 2013]. Associations with the centrosome, centriolar satellites and the mid body might also underlie BBS4 and 6 facilitation of cell cycle regulation and mitosis [Kim et al., 2004, 2005; Zhang et al., 2014]. More recently, there have been reports showing that several BBS proteins enter the nucleus where they can influence gene expression through interactions with the polycomb group member protein RNF2 [Gascue et al., 2012; Scott et al., 2017].

Although our results suggest BBS proteins might have alternative functions independent of each other in different tissues, it is important to mention the evidence that argues against this hypothesis. Overall there is little evidence of a genotype–phenotype relationship among individuals affected with BBS, with a lack of tissue-specific defects in BBS patients carrying mutations in different BBS genes. The one exception here is the renal phenotype. A recent meta-analysis study in the Czech Republic found that the core BBSome subunits BBS2, 7 and 9 manifest as more critical in the kidney [Niederlova et al., 2019]. Similarly, the risk factor for severe renal disease were found to vary between patients harbouring BBS1, 2, 9, 10 or 12 mutations in a detailed study with 350 BBS patients in the UK [Forsythe et al., 2017]. An additional argument is that most functionally tagged BBSome proteins tend to show the same expression pattern (exclusively ciliary localisation) in cultured cells [Barbelanne et al., 2015; Ye et al., 2018]. Lastly, biochemical analyses have shown that the BBSome proteins consistently build one stable functional complex [Nachury et al., 2007; Klink et al., 2017].

In conclusion, we have seen that *Bbs* transcripts are not stoichiometrically expressed in different tissues and that loss of Bbs function affects expression of other transcripts differently. These data support the hypothesis that in some tissues at least, BBS proteins do not only function in a complex but might also have alternative functions in a ciliary independent context independent of one another.

Materials and methods

Animals

All mouse work was performed as per ethical approval from appropriate governing bodies. Experiments were performed in accordance with guidelines provided by Association for Research in Vision and Ophthalmology (ARVO). Animals were maintained on a cycle of 12 hours of light (200 lux) and 12 hours of darkness. The generation and characterisation of *Bbs6*/*Mkks* and *Bbs8*/*Ttc8* knock out (KO) mice have been previously described. For analysis of wild-type tissues, organs were harvested from C57BL/6 mice aged between 6 and 8 months. For comparison between control and *Bbs* knock out tissues, littermate controls of the same age were used in all experiments.

Biological materials

Mice were euthanised by cervical dislocation. Brain, kidney, lung, spleen, heart, oviduct and retina tissue samples were dissected from adult female mice. Tissues were placed immediately in TRIzol Reagent (Thermo Fisher Scientific) (for RNA) or snap frozen (for Western blotting) and stored at −80°C until further use.

RNA isolation

Total RNA was isolated from tissue samples using TRIzol Reagent (Thermo Fisher Scientific). Tissues were homogenised using a FastPrep® -24 classic (MP Biomedicals) bead-basher at a setting of six (6 m/s) for 20–60 s, periodically placing the samples on ice in between pulses. RNA extraction was performed according to manufacturer's instructions and stored at −80°C. RNA quality and quantity were measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) following manufacturer's instructions. Only samples with an $OD_{260/280}$ reading between 1.8 and 2.1 were used for gene qRT-PCR experiments.

Reverse transcription and qRT-PCR

For analysis of target gene mRNA expression, 4 µg of RNA was reverse transcribed into cDNA in a 20 µL reaction volume using the SuperScript™ III first-strand synthesis system (Thermo Fisher Scientific) according to manufacturer's instructions.

Reverse transcription products were diluted in DEPC H_2O. The cDNA was diluted 1:50 or 1:20 while using *Gapdh* or *Bbs* primers respectively. One microliter of the diluted cDNA was used in each qPCR reaction, with a total volume of 10 µl. qRT-PCR amplification was performed using the SYBR® -Green reagent (Life Technologies) on a Step One Plus™ Real-Time PCR machine (Applied Biosystems; Thermo Fisher Scientific,

Inc.). The thermocycler conditions were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s; and a final extension at 72°C for 1 min. mRNA expression of the *Bbs* genes were calculated using the $2^{-\Delta\Delta Ct}$ method. All primer sequences used for qRT-PCR analysis are listed below (Table 1).

Melt curve analysis was performed to assess the amplification of single specific product (Supporting Information Figure 3). Primer amplification efficiency was determined prior to carrying out qPCR analysis (Table 1). Since alternative splicing can produce transcripts with different stabilities, we designed primers to recognise as many of the different BBS transcripts as possible (Table 1).

'ΔCt' is the difference in expression of a gene of interest (*Bbs* genes) and the reference gene, namely *Gapdh*. '1/ΔCt' termed as 'expression factor' was used to show the relative gene expression across tissues. The expression factor (mean of 1/ΔCt values) was used to make the pie charts. The expression factors of BBSome mRNAs (*Bbs1*, *Bbs2*, *Bbs4*, *Bbs5*, *Bbs7*, *Bbs8*, *Bbs9* and *Bbs18*) in different organs were normalised to either brain or heart. The sum of normalised values of all BBSome components in each organ was set as 100%. The percentage expression of individual Bbs gene was calculated and represented as a pie chart. Similarly, all three chaperonin-like gene expression percentages (*Bbs6*, *Bbs10* and *Bbs12*) are plotted as pie charts.

Statistical Analysis

Statistical differences between multiple groups were assessed using ANOVA followed by Dunnett's multiple comparison test (GraphPad Prism 6.0, GraphPad Software, San Diego, CA). Error bars represent the mean ± standard deviation. Results are considered statistically significant if $^{*}p < 0.05$.

Author Contribution

S.R.P. and H.L.M.-S. were responsible for conception and experimental design. S.R.P., A.F., A.-K.V., L.B., S.S. and V.K. carried out experimental work. S.R.P. generated figures. S.R.P. and H.L.M.-S. co-wrote the manuscript.

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Conflict of interest statement

The authors have declared no conflict of interest.

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ORIGINAL ARTICLE

Bardet–Biedl Syndrome proteins regulate cilia disassembly during tissue maturation

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Abstract

Primary cilia are conserved organelles that mediate cellular communication crucial for organogenesis and homeostasis in numerous tissues. The retinal pigment epithelium (RPE) is a ciliated monolayer in the eye that borders the retina and is vital for visual function. Maturation of the RPE is absolutely critical for visual function and the role of the primary cilium in this process has been largely ignored to date. We show that primary cilia are transiently present during RPE development and that as the RPE matures, primary cilia retract, and gene expression of ciliary disassembly components decline. We observe that ciliary-associated BBS proteins protect against HDAC6-mediated ciliary disassembly via their recruitment of Inversin to the base of the primary cilium. Inhibition of ciliary disassembly components was able to rescue ciliary length defects in BBS deficient cells. This consequently affects ciliary regulation of Wnt signaling. Our results shed light onto the mechanisms by which cilia-mediated signaling facilitates tissue maturation.

Keywords Retinal dystrophy · Ciliopathy · Signaling pathways · Proteasomal degradation · Signaling inhibitors

Introduction

Primary cilia are microtubule-based organelles that protrude from the cellular membrane and are anchored by modified centrioles referred to as basal bodies. Cells use primary cilia as specific sensory organelles to detect external cues and mediate intracellular signaling during cell differentiation, organogenesis and tissue homeostasis. Cilia are involved in several signaling pathways including Wingless-related integration site (Wnt), Hedgehog (Hh) and platelet-derived growth factor receptor α (PDGFRα) [1–4], are evolutionally conserved and vital for diverse organisms ranging from metazoans to mammals. Although they are present on almost every cell type [5], not all cells retain their cilium throughout development and adulthood. In many tissues, for example the Organ of Corti in the mammalian cochlea [6], the luminal epithelial cells within mammary gland [7] and corneal endothelial cells in the eye [8], the cilium is vital during development but disassembles upon maturation. However, the physiological consequences of ciliary disassembly are largely unexplored. Cilia can disassemble in response to environmental stress [9], during differentiation or cell cycle progression [10, 11]. This shortening or absorption of the cilium will inevitably influence its signaling and functional role [12–16].

The importance of cilia during organogenesis and function of the vertebrate eye is well documented, particularly in photoreceptor cells [17, 18]. However, we only recently showed that the retinal pigment epithelium (RPE), a pigmented monolayer epithelium essential for photoreceptor development and visual function, also relies on the primary cilium for maturation through the regulation of canonical Wnt signaling [19]. Numerous reports have shown that RPE-derived cell lines are ciliated in vitro [20, 21] and although ciliary assembly and disassembly pathways have been studied in these models, the precise mechanisms are still being determined. The effect of these processes on downstream

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signaling pathways has not been established [22, 23]. Moreover, RPE ciliogenesis and ciliary disassembly have not been investigated in vivo. Although reports have suggested that cilia in the RPE are retained throughout adulthood [24], a report from Nishiyama suggested that they disappear in the adult rat RPE [25]. Therefore, we set out to investigate the extent of ciliation in developing vertebrate RPE and to understand the molecular mechanisms underlying ciliary disassembly during development.

Various ciliary proteins including Bardet–Biedl Syndrome (BBS) and Nephronophthisis (NPHP) proteins show ciliary localization [26] and regulate cilia length [27, 28]. BBS proteins have been shown to direct ciliary trafficking, whereas many of the NPHP proteins function as ciliary gatekeepers [26, 28–31]. Certain mutations in the genes encoding these proteins can cause severe ciliopathy phenotypes including retinal degeneration, cystic kidneys, central obesity and situs inversus [32, 33].

Ciliary disassembly is known to be regulated by various cell cycle regulators. This process is mediated by human enhancer of filamentation 1 (HEF1/NEDD9) [34], which translocates from focal adhesions to the basal body, a structure derived from the mother centriole at the base of the cilium. Translocation of the scaffold protein HEF1 activates Aurora A kinase (AurA), which in turn activates histone deacetylase 6 (HDAC6), destabilizing the microtubule axoneme and thereby causing ciliary disassembly [22]. Inversin (NPHP2) influences ciliary disassembly by regulating AurA [35] and Wnt signaling [36]. BBSome-interacting protein 1 (BBIP10/BBS18), an accessory BBS protein, has been shown to directly interact with HDAC6, thereby modulating acetylation and stabilization of cytoplasmic microtubules [37]. BBS proteins have also been shown to modulate Wnt signaling via degradation of β-catenin [19, 38, 39], which is controlled by precise coordination of phosphorylation and dephosphorylation events [40, 41].

In this study, we investigated the influence of ciliary disassembly components in the developing RPE in vivo and in vitro. Furthermore, we sought to elucidate how BBS proteins mediate ciliary disassembly and how this influences downstream signaling cascades involved in tissue morphogenesis.

Results

Primary cilia are transiently expressed in the developing RPE

We characterized primary cilia expression in mouse flat-mount RPE at different stages of development using immunohistochemistry, RNA expression and electron microscopy. An antibody against ADP Ribosylation Factor-Like GTPase 13B (Arl13b) was used to identify primary cilia. Co-staining with other cilia (acetylated α-tubulin) and basal body markers (Pericentrin2, GT335) confirmed Arl13b as a reliable cilia marker in the RPE (Fig. 1a, b; Supp. Figure 1a–d). Tight junction marker Zonula occludens-1 (ZO-1) (Fig. 1a, b; Supp. Figure 1b, c) and β-catenin were used as membrane markers to visualize cell borders (Supp. Figure 1d). We performed stainings at various time points from E14.5 onward and observed that the expression of primary cilia in the developing RPE diminishes as the RPE matures (Fig. 1c–f). E14.5 was the earliest time point at which cilia were reliably observed and E18.5 was chosen instead of post-natal stages in order to circumvent embryonic lethality. The highest percentage of ciliated cells was observed at E14.5 and E16.5 (69.8 and 64%, respectively). By E18.5, only 26% of cells in the RPE were ciliated, suggesting that the primary cilium had retracted in the majority of the cells (Fig. 1f). Changes in cilia length accompanied cilia retraction, decreasing from 2.63 µm at E16.5 to 0.95 µm at E18.5 (Fig. 1g–j). In support of developmentally dependent transient expression of the primary cilium, we also observed differences in ciliation between the center and periphery of the RPE (compare Fig. 1c–e and Supp. Figure 1e–h). Ciliated cells were rarely identified after E18.5. In P1 and adult RPE, only a few cells could be identified with short stumpy Arl13b and acetylated α-tubulin positive cilia (Fig. 2a; Supp. Figure 1i–k). As previously documented [42], we also observed a developmental increase in cell size (Fig. 1c–e, Supp. Figure 1e–g). Because many cells were observed with cilia at embryonic timepoints, these data suggest that the primary cilium retracts as the RPE develops.

To confirm retraction of the primary cilium in mature RPE tissues, we prepared transmission electron micrographs (TEM) of mouse RPE at multiple time points and imaged all identifiable basal body or ciliary axoneme profiles. All profiles were classified into one of the three categories that we defined as follows: basal bodies or centrioles with no attached membrane vesicles were categorized as Class I, whereas Class II profiles were identified as basal bodies containing a membranous attachment (the ciliary vesicle). Class III profiles were those with an extended ciliary axoneme into the extracellular space (Fig. 2b). Class I profiles are likely over-represented as an artifact of sectioning through a basal body of a Class II and III structure that had an attached membrane or axoneme in a different cutting plane. Consistent with an increase in ciliary disassembly, the number of Class III profiles decreased with RPE maturation (Fig. 2c). Combined, these data are consistent with our observations on immunohistochemistry and imply that primary cilia disassemble upon maturation of the RPE.

To further support retraction of the primary cilium, we measured changes in gene expression of two ciliary genes, *Arl13b* and *Ift88* in isolated mouse RPE cells using

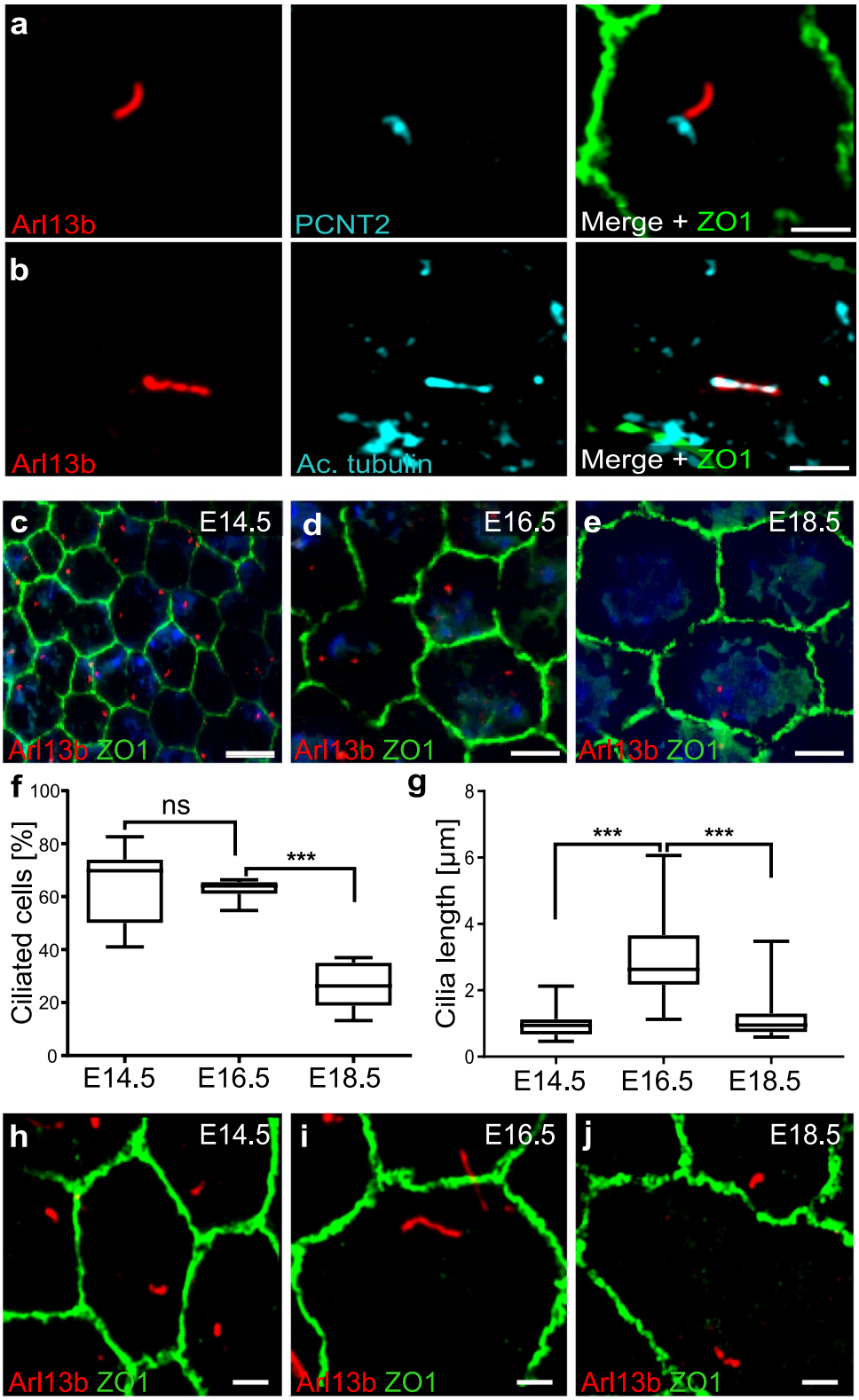


Fig. 1 The primary cilium is transiently expressed during RPE development. Representative high-resolution immunofluorescence images of E16.5 mouse RPE flatmounts labeled with antibodies against ciliary structures show co-localization of Arl13b and acetylated α-tubulin extending from the basal body (**a**, **b**). Arl13b (axoneme marker, red); Pericentrin 2 (PCNT2, basal body marker, cyan); acetylated α-tubulin (Ac. tubulin, axoneme marker, cyan); Zona Occludens (ZO-1, cell junctions, green). Low magnification immunofluorescence images show ciliation (number of ciliated cells) at three embryonic timepoints (**c**–**e**). Boxplots show a significant decrease in the number of ciliated cells from E14.5 to E18.5 (**f**). E14.5 $n=1700$ cells, E16.5 $n=750$ cells, E18.5 $n=650$ cells. Boxplots of cilia length demonstrate that mouse RPE cilia are longest at E16.5 (**g**). $n=25$ for each age group. High-resolution immunofluorescence images of cilia (Arl13b, red) highlight differences in ciliary length between E14.5 and E18.5 (**h**–**j**) as quantified in **g**. Three or more animals were used per data set. Statistics were done using the Dunnett's multiple comparison test $^{***}p \leq 0.001$; *ns* not significant. Scale bars: **a**, **b**, **h**, **i**, **j** 2 µm; **c**–**e** 10 µm

quantitative real-time PCR (qRT-PCR). We developed a method to isolate pure RPE cells from embryonic mouse eyes and confirmed cell purity by determining lack of choroidal or retinal-specific gene expression (Supp. Figure 2a–c). *Gapdh* was taken as the housekeeping control as its expression remained stable from E14.5 through to P7 in control and knockout mouse RPE (Supp. Figure 2d, e). Expression of both cilia genes peaked at E16.5 and decreased as the

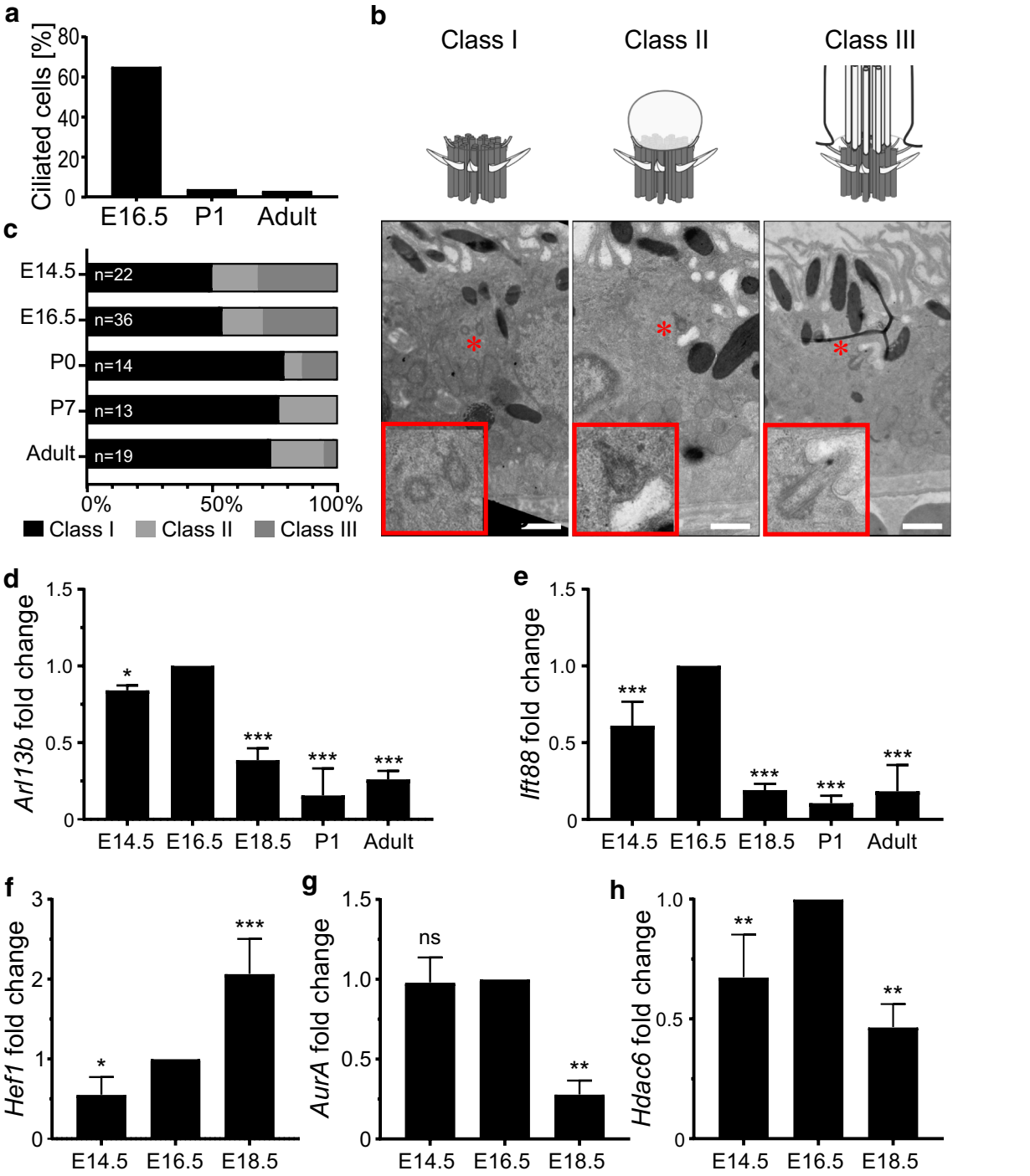


Fig. 2 Primary cilia disassemble during mouse RPE development. Quantification of ciliation in mouse RPE at E16.5, post-natal day 1 (P1) and adult show that the number of ciliated cells drastically decreased after birth (**a**). Transmission electron micrographs of basal bodies or ciliary axoneme profiles (marked by red asterisks) of adult mouse RPE (**b**). Schematics show classification of ciliary structures into different classes (I, II and III), depending on the absence (class I) or presence of a membranous attachment (the ciliary vesicle) (class II) or ciliary axoneme (class III). Quantification of class I-III profiles shows that the number of class III profiles decrease as the RPE matures (**c**). Gene expression as measured by quantitative real-time PCR from isolated primary mouse RPE cells show altered expression of ciliary (*Arl13b* and *Ift88*) and ciliary disassembly markers (*Hef1, AurA* and *Hdac6*) (**d**–**h**). Fold changes and significance were calculated relative to E16.5. Values represent data from three or more independent animals (biological repeats) each with three technical repeats per data set. Statistics were done using the Dunnett's multiple comparison test $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; *ns* not significant. Scale bars: 2 µm

tissue matured (Fig. 2d, e). Because ciliary retraction in the RPE may be actively controlled via ciliary disassembly, we also analyzed the gene expression of *Hef1, AurA* and *Hdac6* [22, 43, 44], which are known regulators of ciliary disassembly. Expression of ciliary disassembly components changed dynamically as the cilium retracted (Fig. 2f–h). *Hef1* expression increased gradually from E14.5 to E18.5. Expression of *AurA* and *Hdac6* was decreased by ~ 50% between E16.5 and E18.5. This suggests that *AurA* and *Hdac6* expressions are highest just before ciliary disassembly is observed and decline by E18.5, at which point the majority of cilia have disassembled.

Taken together, these data demonstrate that the primary cilium is disassembled upon maturation of the RPE. Temporal and spatial patterning of ciliation across the RPE matches the known mechanism of maturation for this tissue. The present data and our previous work [19] support the hypothesis that the transient expression of the primary cilium is required for RPE maturation, which occurs in an organized pattern. To further understand the mechanism of ciliary disassembly, we next sought to investigate how ciliary proteins contribute to the process of ciliary disassembly.

Loss of BBS proteins affects ciliated RPE

We focused on Bardet–Biedl syndrome (BBS) proteins in view of our prior observation that BBS proteins regulate primary cilia length and ciliary trafficking [27]. BBS6 (MKKS) is a component of the BBS chaperonin complex thought to be required for BBSome assembly. BBS8 (TTC8) is a component of the BBSome complex required for ciliary trafficking. We observed that RPE tissue from *Bbs6* knockout (*Bbs6*$^{-/-}$) mouse embryos displayed fewer ciliated cells at E16.5. The knockout cilia were shorter (0.87 ± 0.67 µm) compared to WT (2.25 ± 0.94 µm) littermates (Fig. 3a–d). In addition to changes in cilia length, we also observed changes in cell size in *Bbs6* knockout mouse embryos. In WT, we saw little variation in cell size between neighboring cells. However, in *Bbs6*$^{-/-}$ we observed an increase in average cell size and broader distribution of cell size (Fig. 3a; Supp. Figure 3a, b). This abnormal patterning reflects disrupted RPE maturation as we had seen for *Bbs8*$^{-/-}$ in our previous paper [19]. Abnormal patterning is possibly a consequence of dysregulation of cell junction components such as Occludin and Epithelial Cadherin (Cadherin 1). Occludin is an integral membrane protein found at tight junctions [45], and Epithelial Cadherin [46] is a major component of adherens junctions, both of which are essential for the regulation of RPE intercellular junction integrity and function, and apical basal polarity. Ras-related protein Rab-27A (Rab27) and Myosin VIIA (Myo7a) are both required for melanosome transport in the RPE, dysfunction of which leads to defective visual cycle [47]. Expression of these genes is seen as a marker and benchmark for RPE maturation and their disruption can contribute to aberrant epithelial morphology. We observed a downregulation of *Cdh1, Ocln*, *Rab27a* and *Myo7a* as quantified by qRT-PCR (Supp. Figure 3c). *Bbs8* knockout (*Bbs8*$^{-/-}$) embryos also showed reduced number of ciliated RPE cells at E16.5 compared to that of WT (Fig. 3e, f, Supp. Figure 3c) although less significant changes in gene expression were observed.

To further elucidate the mechanisms by which BBS molecules regulate ciliary disassembly, we utilized an in vitro culture system by knocking down (KD) BBS8 or BBS6 using short interfering RNA (siRNA) in hTERT-RPE1 cells. This cell line has previously been used to characterize ciliary disassembly and is derived from human RPE tissue. Knockdown was validated by RT-qPCR and Western blotting (Supp. Figure 4a–d). Cells were serum-starved to induce ciliogenesis. Similar to the in vivo mouse RPE, we observed fewer ciliated cells upon BBS8 and BBS6 KD (24.9 ± 11.6 and 24.1 ± 9.62%) compared to the non-targeting siRNA control (NTC, 74.7 ± 9.77%) (Fig. 3g, h). In the remaining cells that retained cilia, cilia length was also significantly reduced compared to NTC (NTC: 3.58 µm, BBS8 KD: 2.26 µm, BBS6 KD: 2.62 µm) (Fig. 3i).

Interaction of BBS proteins with mediators of ciliary disassembly

Next, we sought to identify whether additional BBS proteins interact with Inversin, a protein known to be involved in AurA mediated ciliary disassembly [35]. Since we had previously shown that BBS8 directly interacts with Inversin during regulation of Wnt signaling in development of the RPE [19], we wanted to see if this interaction extended to other BBS proteins. Using GFP-traps, we were able to pull down overexpressed BBS6-myc and BBS2-myc with Inversin-eGFP in HEK293T cells (Fig. 4a, b). To confirm the physical interaction between Inversin and BBS6 in situ, a proximity ligation assay (PLA) was performed using antibodies against endogenous Inversin and overexpressed myc-BBS6. Positive PLA events shown as distinct fluorescent foci confirmed the interaction (Fig. 4c).

In an immortalized murine kidney medullary (KM) cell line, Inversin is localized to the base of the cilium as seen by Inversin co-localization with acetylated α-tubulin in the majority of ciliated cells (Fig. 4d, e). These cells were used as endogenous Inversin is better detectable in these cells upon immunocytochemistry. Furthermore, the Wnt signaling and ciliary phenotype are recapitulated in these cells [27]. In KM cells derived from *Bbs6* knockout (*Bbs6*$^{-/-}$) mice, significantly fewer cilia showed Inversin localization at the base, and Inversin expression was more dispersed throughout the cytoplasm (Fig. 4d, f). Combined these data suggest

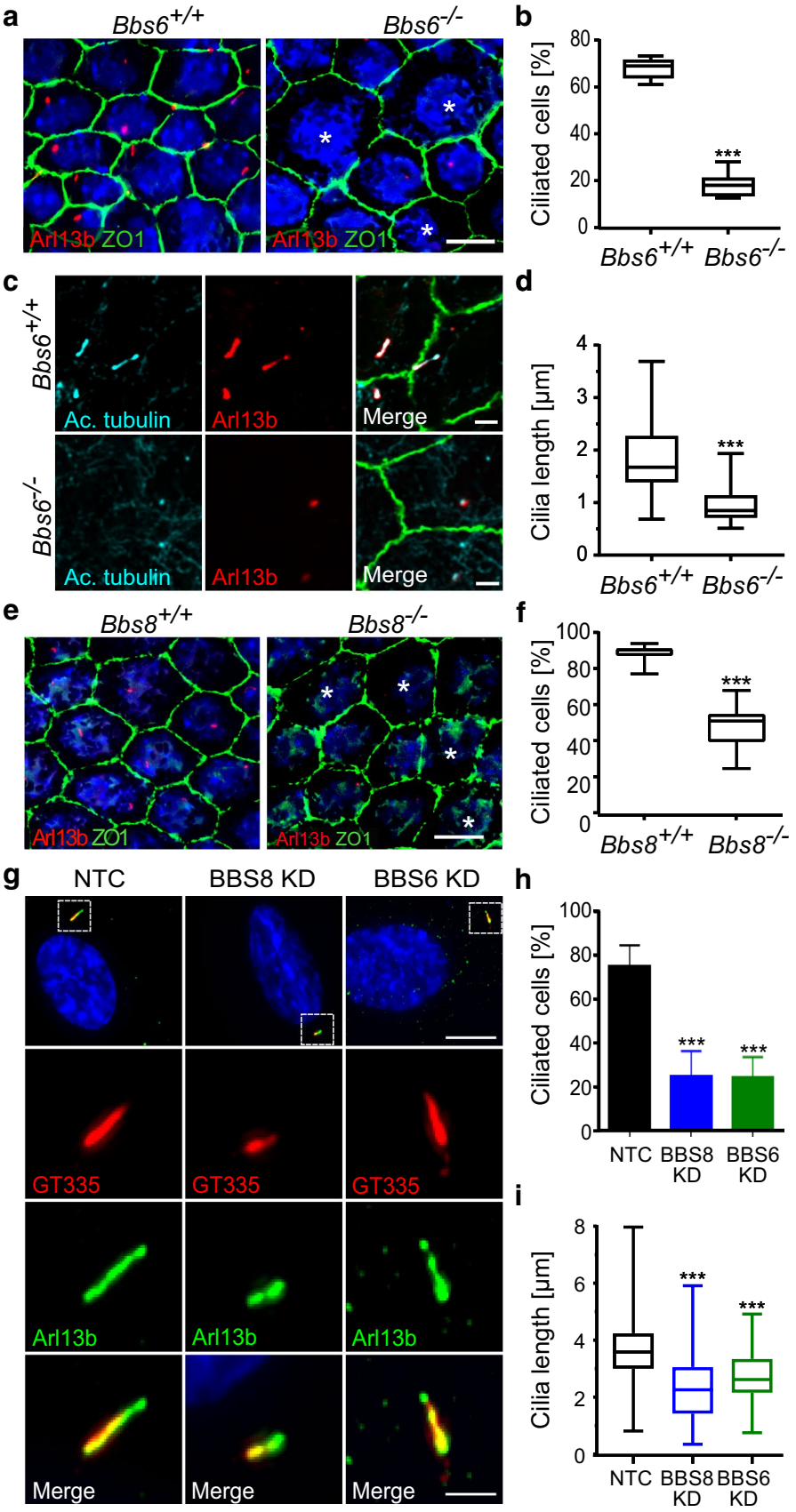


Fig. 3 Absence of BBS proteins decreases RPE ciliation in vivo and in vitro. Representative immunofluorescence images of E16.5 mouse RPE flatmounts from wildtype and *Bbs6*$^{-/-}$ littermates, labeled with antibodies against the primary cilium (Arl13b; red, acetylated α-tubulin (Ac. tubulin); cyan) and cell junctions (Zona Occludens (ZO-1); green) (**a**, **c**). Boxplots of ciliation (**b**) and cilia length (**d**) show a significant decrease in cilia number and cilia length in *Bbs6* knockout animals. Representative immunofluorescence images of E16.5 mouse RPE flatmounts from wildtype and *Bbs8*$^{-/-}$ littermates, labeled with antibodies against Arl13b; red and ZO-1; green show a reduced number of ciliated cells in *Bbs8* knockout mice (**e**). Boxplots confirmed the significant reduction of ciliated cells in *Bbs8* knockout RPE (**f**). Representative immunofluorescence images of primary cilia labeled with antibodies against Arl13b; green and polyglutamylated tubulin (GT335); red in BBS8 and BBS6 KD hTERT-RPE1 compared to non-targeting control (NTC) (**g**). Graphical representation of percentage of ciliated cells (NTC $n=250$, BBS8 KD $n=150$ and BBS6 KD $n=200$) (**h**) and cilia length (**i**) of control in comparison to KD cells. White asterisks (*) label cells lacking cilia. Three or more individual animals were used per sample set. Statistical analyses in **b**, **d** and **f** were performed using two-tailed Mann–Whitney *U* test, where $***p<0.001$. For **d** $n=40$ cells per genotype. Statistical analyses in **h** and **i** were done using the Dunnett's multiple comparison test $***p\leq 0.001$. Scale bars: **a**, **e** 10 µm; **b** 2 µm; **g** 10 µm, magnified images 5 µm. *KD* Knockdown

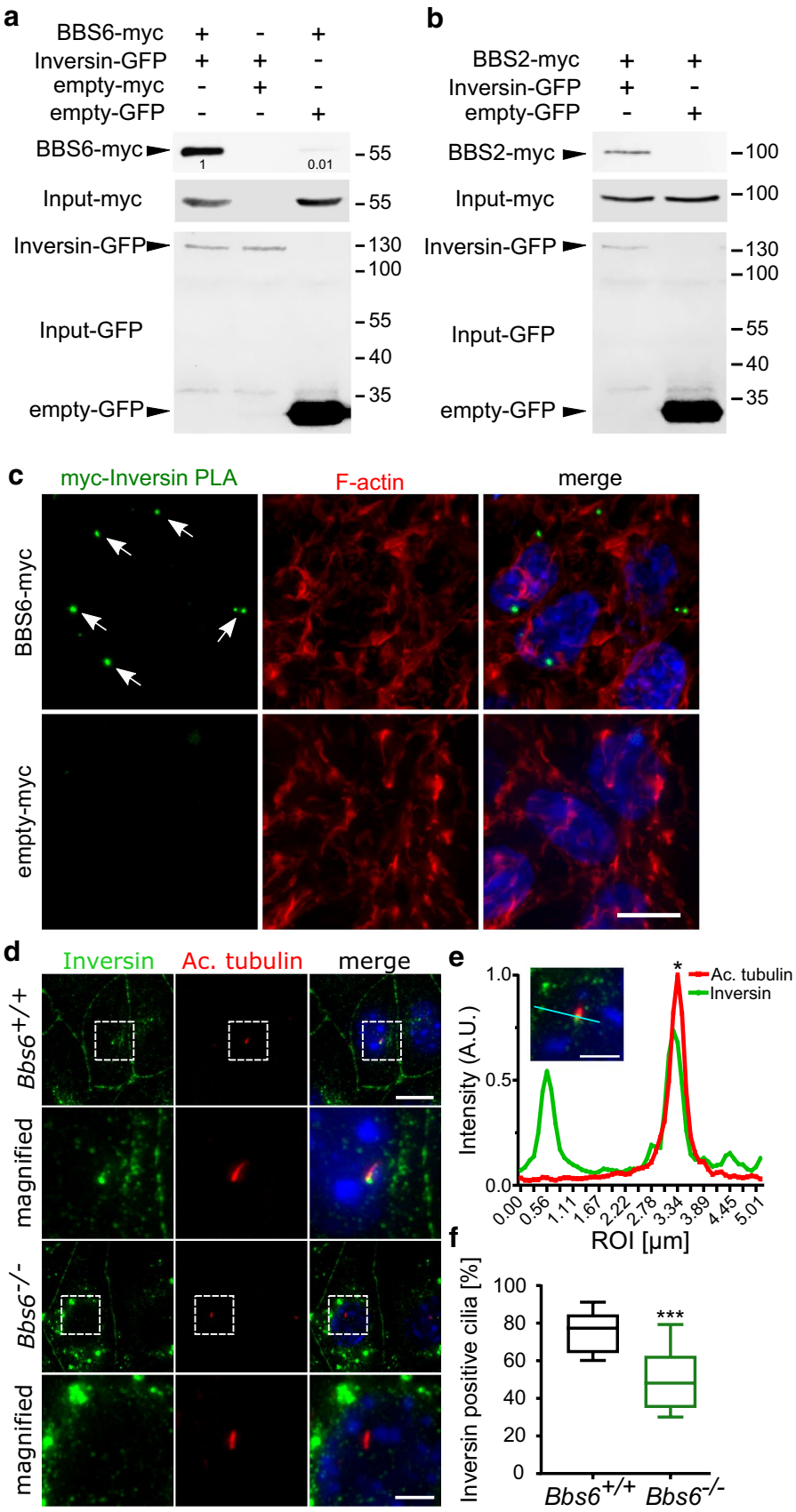
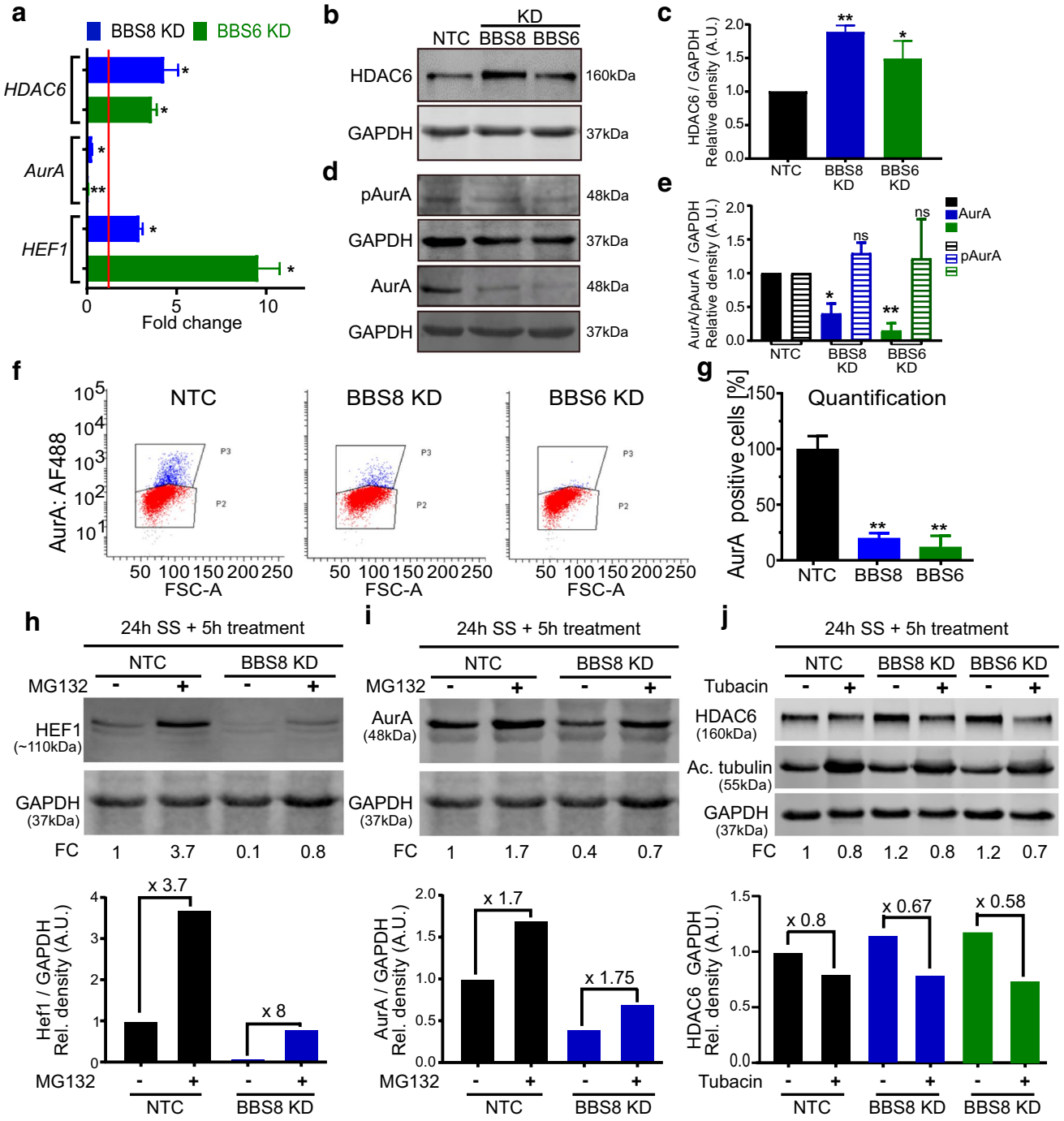


Fig. 4 Mislocalization of Inversin upon loss of BBS proteins. HEK293T cells transiently co-transfected with *myc*-tagged *BBS* and *Inversin-GFP* plasmids. Cell lysates subjected to GFP-TRAP pulldown followed by Western blotting show that Inversin-GFP interacts with myc-tagged BBS6 (**a**) and myc-tagged BBS2 (**b**). Proximity ligation assays (PLA) of HEK cells overexpressing *myc-BBS6* were performed using antibodies against myc and endogenous Inversin. Positive PLA foci (green) indicate interaction between Inversin and BBS6. Empty-myc transfected control cells did not display positive PLA foci. TRITC-Phalloidin (F-actin, red) was used to visualize cell outlines (**c**). Representative immunofluorescence images of kidney medullary (KM) cells labeled with antibodies against the primary cilium (acetylated α-tubulin (Ac. tubulin); red) and Inversin (green) show diminished localization of Inversin to the base of the cilium in $Bbs6^{-/-}$ cells as compared to the wildtype control (**d**). ROI linear profile represents fluorescence intensity of corresponding cyan line on the merged image. Peaks indicated by asterisk represent area of co-localization of Inversin and Ac. tubulin in wildtype KM cells (**e**). $Bbs6^{+/+}$ $n=215$ cilia, $Bbs6^{-/-}$ $n=140$ cilia. Boxplots show a reduction in percentage of Inversin positive Ac. tubulin in $Bbs6^{-/-}$ KM cells compared to control cells (**f**). Statistical analyses in e were performed from three independent experiments using two-tailed Mann–Whitney *U* test, where $***p<0.001$. Scale bars: **c**, **d** 10 µm



that BBS6 and BBBS2 proteins interact with Inversin and regulate its expression at the ciliary base.

BBS proteins regulate key mediators of primary ciliary disassembly

In mammalian cells, ciliary disassembly is mediated by recruitment of HEF1 at the basal body, leading to downstream phosphorylation and activation of AurA and HDAC6, and subsequent destabilization of ciliary tubulin [22, 48]. We wanted to determine whether BBS proteins are involved in cilia maintenance by protecting the cilium against AurA-HDAC6-mediated disassembly. Gene expression analysis showed an increase of ciliary disassembly genes *HDAC6* and *HEF1* upon knockdown (KD) of BBS8 or BBS6, consistent with reduced ciliary length (Figs. 3 g–i; 5a). Western blot analysis also showed elevated levels of HDAC6 protein expression in hTERT-RPE1 cells (Fig. 5b, c). Expression of

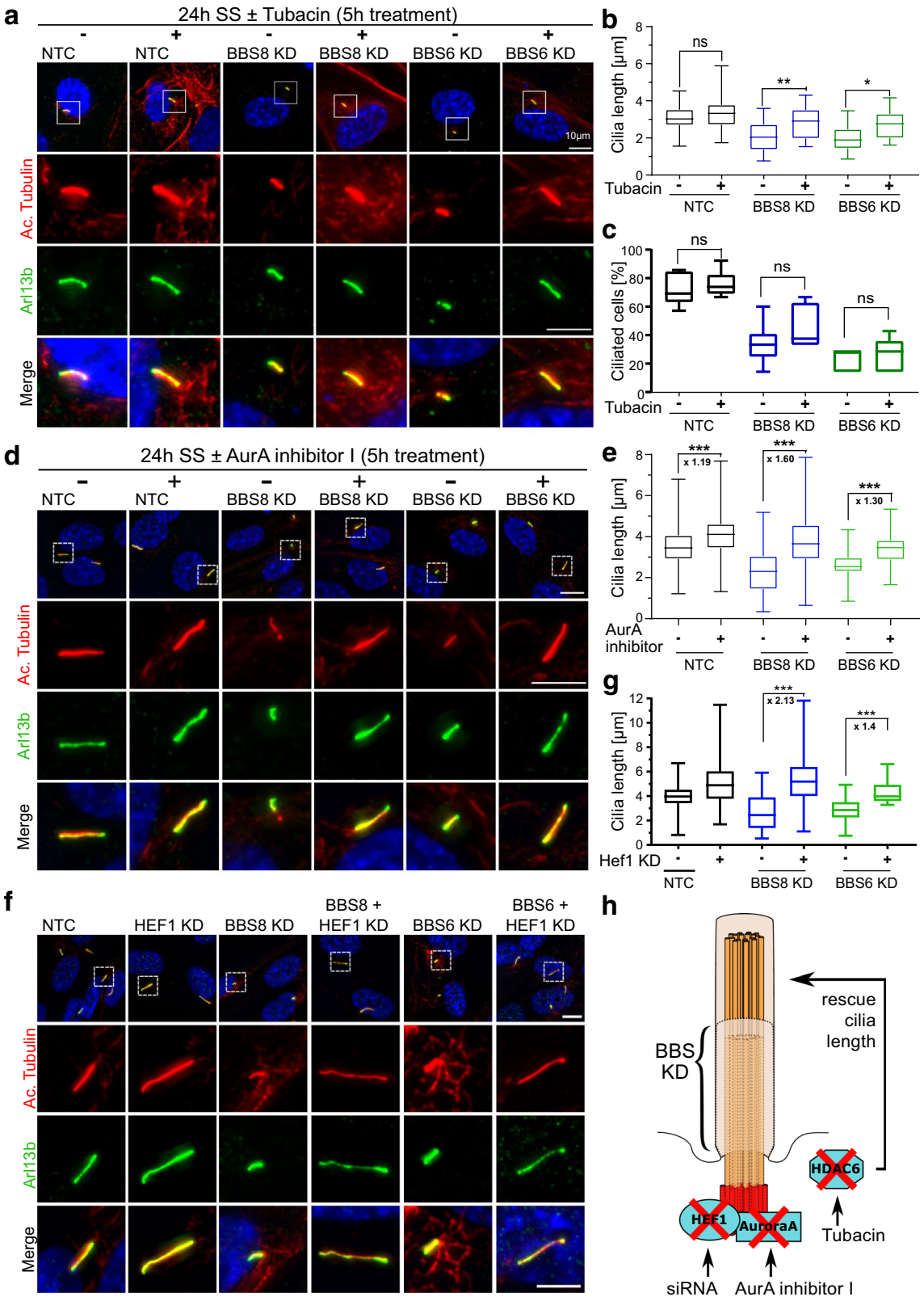
◂**Fig. 5** BBS proteins regulate key mediators of cilia disassembly. Quantitative real-time PCR shows increased gene expression of cilia disassembly components (*HEF1* and *HDAC6*) and decreased expression of *AurA* relative to non-targeting control (NTC, red line) in serum-starved BBS8 or BBS6 knockdown (KD) hTERT-RPE1 cells. *GAPDH* was used as housekeeping control (**a**). Western blots show a significant increase in protein levels of HDAC6 upon KD of BBS8 and BBS6 in serum-starved hTERT-RPE1 cells (**b**, **c**). Conversely, AurA protein levels were decreased upon KD of BBS8 and BBS6 in serum-starved hTERT-RPE1 cells, although pAurA levels were retained (**d**, **e**) suggesting an increased ratio of active over total AurA. Flow cytometry analysis was used to further quantify AurA expression in serum-starved BBS8 and BBS6 KD hTERT-RPE1 cells (**f**, **g**). Representative flow cytometry dot plots show the AurA-positive cell population (P3, blue) and AurA-negative cell population (P2, red) (**f**). Quantification of the AurA-positive cell population confirmed a significant decrease in the BBS8 and BBS6 KD cells compared to NTC (**g**). Western blots show that KD of BBS8 in hTERT-RPE1 cells leads to decreased level of HEF1, which was partially restored by treatment with proteasome inhibitor MG132 (**h**). An 8-fold increase in HEF1 protein expression was observed upon BBS8 KD compared to 3.7-fold in NTC. Decreased protein levels of AurA in BBS8 KD hTERT-RPE1 cells were also partially restored by treatment with proteasome inhibitor MG132 (**i**). Increased levels of HDAC6 in BBS8 KD hTERT-RPE1 cells were concomitant with an increase in acetylated α-tubulin and were reduced upon treatment with HDAC6 inhibitor tubacin, as quantified by Western blot (**j**). Quantification of Western blot data was normalized to GAPDH levels. Bar charts show relative protein expression in arbitrary units (AU). Data are expressed as mean ± SD, n = 3 separate experiments for (**a**-**g**), while $n = 2$ for h-j. Statistical analyses in c, g were done using the Dunnett's multiple comparison test and e using Shidak's multiple comparison test. In **c**, **e** and **g** $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; *ns* not significant. *KD* Knockdown, *NTC* non-targeting control, *SS* serum-starved, *FC* fold change, *FSC-A* forward scatter area

total *AurA* mRNA and AurA protein was decreased in KD cells, as analyzed by qPCR, Western blot and flow cytometry (Fig. 5a, d, e, f, g). In contrast, the level of active phosphorylated AurA (pAurA) was unchanged as shown by Western blot with an antibody specific for pAurA (Thr288) (Fig. 5d, e). Therefore, the ratio of pAurA/AurA was elevated in the KD cells compared to control even without ciliary disassembly via serum activation. Previous reports showed that levels of pAurA peak when cilia disassemble [23]. When we do combined serum activation with BBS KD, we observed a similar trend in that active pAurA is retained upon BBS8 or BBS6 KD (Supp. Figure 5a). As quantified by qPCR, *HEF1* transcript expression was elevated upon both BBS8 and BBS6 KD (Fig. 5a). In an attempt to verify these data in vivo, we analyzed mRNA expression of *Hef1*, *AurA* and *Hdac6* in knockout mouse tissue using unpurified RPE (RPE and choroid). Expression of ciliary disassembly genes *Hef1* and *Hdac6* was increased in $Bbs8^{-/-}$ relative to wildtype control littermate tissue. No significant change in expression level could be seen for *AurA*. In $Bbs6^{-/-}$ mice, *Hef1* was significantly increased, *AurA* significantly decreased and *Hdac6* remained unchanged (Supp. Figure 3d). These gene expression patterns show a similar trend to the in vitro data. Although *HEF1* mRNA expression levels were elevated in vitro and in vivo, Western blot analysis showed a reduction in protein upon loss of BBS8 (Supp. Figure 5b). We hypothesized that in the absence of BBS8, HEF1 is actively phosphorylated and targeted for proteasomal degradation. To test this, we treated serum-starved cells with the proteasome inhibitor MG132. This resulted in ~ 8-fold recovery of HEF1 expression in treated KD cells compared to untreated KD cells which only had a 3.7-fold recovery (Fig. 5h). Therefore, over 50% more HEF1 was recovered in KD cells compared to control (0.1–0.8 vs 1–3.7). Although we saw a mild recovery of total AurA upon inhibiting proteasomal activity in these cells (Fig. 5i), there was little difference between KD and control. Based on these observations, we propose that ciliary disassembly components are differentially regulated by BBS proteins. While loss of BBS8 results in increased levels of HDAC6, it also results in the proteasomal degradation of HEF1 and to some extent of AurA. We wanted to further elucidate whether the ciliary length defect observed in BBS deficient cells could be attributed to dysfunction of ciliary disassembly components.

Ciliary disassembly component inhibition rescues the ciliary length defect caused by BBS knockdown

To confirm that ciliary defects upon BBS KD can be attributed in part to an increase in HDAC6 expression, we treated serum-starved NTC and BBS KD cells with tubacin, a specific HDAC6 inhibitor [22, 49]. Tubacin treatment caused a decrease in HDAC6 and a concomitant increase in the protein level of acetylated tubulin in both NTC and KD cells compared to the vehicle (DMSO) alone, as seen by Western blot (Fig. 5j). This corresponded to a rescue in cilia length (Fig. 6a, b). A significant change in the number of ciliated cells was not observed after treatment with tubacin (Fig. 6c). Next, we used a potent and selective inhibitor of AurA, AurA Inhibitor I [35, 50]. Similarly, this also ameliorated the cilia length defect in BBS8- and BBS6-deficient cells (Fig. 6d, e). To test whether HEF1 activity also underlies the AurA and HDAC6 mediated ciliary disassembly in BBS KD cells, we performed double KD experiments. We observed an appreciable rescue in cilia length in the BBS and HEF1 double KD cells compared to single KD (Fig. 6f, g). We confirmed that double KD did not lead to loss of individual gene KD efficiency (Supp. Figure 4e–g). Recovery of cilia length after inhibition or KD of HEF1, AurA and HDAC6 supports our hypothesis that BBS proteins are involved in the control of ciliary disassembly (Fig. 6h).

To address off-target effects of siRNA we knocked down BBS8 from hTERT-RPE1 cells using single (siRNA 1 or siRNA2) and double siRNAs (siRNA 1 + 2) and looked for levels of HDAC6 via Western blotting. We observed a similar increase in HDAC6 levels in single vs double KD



cells, as quantified using Western blotting analysis (Supp. Figure 5c). Furthermore, using different combinations of siRNA against BBS8 from different companies had the same effect on AurA expression levels (Supp. Figure 5a). Therefore, we believe that the phenotype we observe is specific. To rule out differences in cell cycle stages, which

◂**Fig. 6** Inhibition of ciliary disassembly components rescues BBS mediated ciliary disassembly. Representative immunofluorescence images of cilia stained with antibodies against acetylated α-tubulin (Ac.tubulin, red) and Arl13b (green), from non-targeting control (NTC), BBS8 and BBS6 knockdown (KD) hTERT-RPE1 cells treated with and without HDAC6 inhibitor, tubacin (**a**). Quantification of ciliary length show that tubacin treatment did not affect ciliary length in control, yet was able to increase ciliary length in BBS8 and BBS6 KD cells (**b**). Quantification of ciliated cells showed that treatment with tubacin had no effect on ciliation (**c**). Treatment with AurA inhibitor I was also able to significantly increase ciliary length in BBS8 and BBS6 KD cells. BBS8 and BBS6 KD tubacin-treated cells show a greater increase in cilia length in comparison to their DMSO mock-treated counterparts, while control cells showed minimal increase (**d**, **e**). KD of HEF1 increased ciliary length in control hTERT-RPE1 cells. Double KD of HEF1 and BBS8 or BBS6 was able to reverse the ciliary disassembly phenotype observed in BBS8 or BBS6 KD (**f**, **g**). A model showing the inhibition or KD of ciliary disassembly components causes rescue of cilia length in BBS KD hTERT-RPE1 cells (**h**). Statistical analyses in **b**, **c**, **e**, **g** were done using the Sidak's multiple comparison test from two independent experiments. The length of at least 1000 cilia from the six different treatment groups (NTC treated, untreated, BBS8/6 KD treated, untreated) in each experiment (Tubacin treatment, AurA treatment and Hef1 KD) were measured. $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$; *ns* not significant. *KD* Knockdown, *NTC* non-targeting control, *SS* serum-starved

could affect changes in ciliary disassembly components, we performed cell cycle analysis via fluorescence-activated cell sorting (FACS) using propidium iodide (PI). This corroborated that the majority of cells remained in G0/G1 phase in both NTC and KD cells (Supp. Figure 5d), confirming that our results were cell cycle independent.

Loss of BBS proteins regulate HDAC6, thereby influencing post-translational modification of β-catenin

Previous studies have shown that ciliogenesis and ciliary disassembly modulate the switch from β-catenin-dependent canonical to non-canonical Wnt signaling pathways [36, 51]. Therefore, we examined the impact of BBS-regulated ciliary disassembly components on Wnt signaling. β-catenin is a direct substrate of HDAC6, which deacetylates β-catenin at lysine residue (K49), inhibiting downstream phosphorylation [52]. This results in β-catenin stabilization and nuclear accumulation. Although we and others have shown that suppression of *BBS* genes results in stabilization of β-catenin and altered regulation of downstream Wnt targets [18, 38], the mechanisms that cause this stabilization are not yet known. Loss of BBS is not thought to directly affect the core activity of the proteasome [38], which suggests a defect in phosphorylation and subsequent targeting of β-catenin for degradation. Since the stability of β-catenin is mediated by various specific phosphorylation and acetylation events at different sites, we hypothesized that upon loss of BBS, β-catenin becomes differentially modified. Consistent with this, the level of acetylated β-catenin (Lys49) was reduced after BBS8 or BBS6 KD in hTERT-RPE1 cells as quantified by Western blotting (Fig. 7a). We also sought to identify further downstream phosphorylation events of β-catenin that lead to degradation. Using phospho-specific antibodies, we detected a decrease in phosphorylation at T41/S45 and S33/37/T41 as a result of BBS8 and BBS6 KD (Fig. 7b, c).

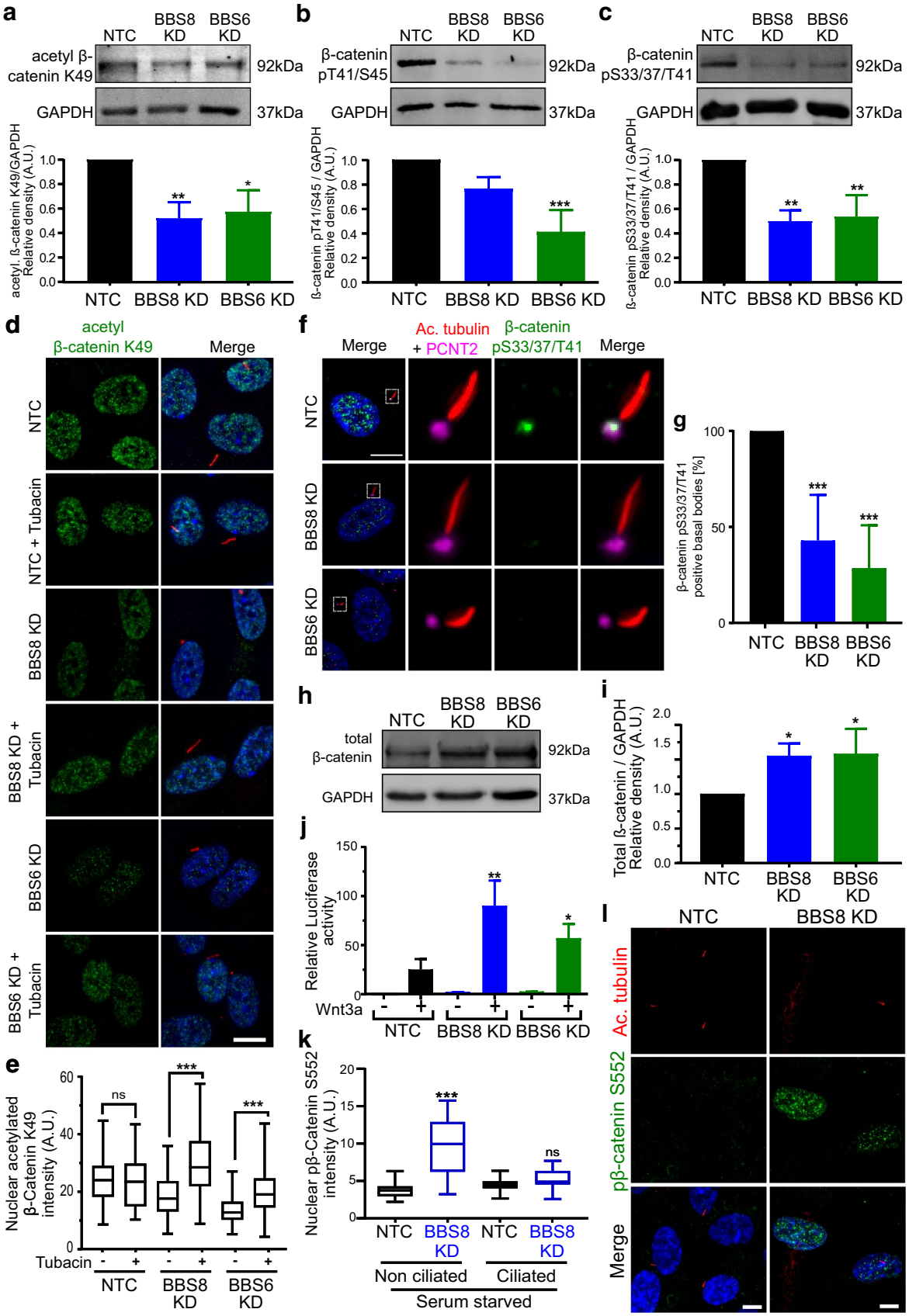
To strengthen our findings on Western blot, we performed immunocytochemistry using an antibody against acetylated β-catenin (Lys49) and observed a reduction in nuclear fluorescence intensity upon BBS KD. Treatment with HDAC6 inhibitor tubacin rescued the levels of acetylated β-catenin K49 in BBS-deficient cells (Fig. 7d, e). Previous studies showed that phospho-(S33/37/T41)-β-catenin localizes to the base of the cilium [51, 53]. We observed localization of phospho-(S33/37/T41)-β-catenin not only to the basal body, but also in the nucleus (Fig. 7f). Upon KD of BBS8 and BBS6, this nuclear localization is diminished while localization at the basal body is often absent (Fig. 7f, g).

Reduction of phosphorylation at S33/37/T41 ultimately results in reduced degradation and consequent increase in levels of total β-catenin as observed in Western blot (Fig. 7h, i). This increased level of total β-catenin translated to increased levels of β-catenin activity as measured via luciferase assays (Fig. 7j). Stimulation with Wnt3a conditioned medium significantly increased β-catenin driven TCF/LEF transcription after BBS8 or BBS6 KD. In contrast to phosphorylation at S33/37/T41, phosphorylation at S552 causes stabilization and nuclear accumulation of β-catenin [54]. Consistent with this, and the increased activity of β-catenin, we detected elevated expression and nuclear accumulation of pS552 β-catenin in nonciliated BBS8 KD cells (Fig. 7k, l). Together, these experiments show that loss of BBS molecules influences post-translational modification of β-catenin, which ultimately regulates its signaling activity.

Discussion

In the present work, we demonstrate that ciliary trafficking proteins are required for homeostasis of primary ciliary disassembly components, specific regulation of which is required for ciliary retraction and regulation of signaling pathways. Precise regulation of ciliation is an absolute requirement for tissue differentiation [8, 55]. We focused our attention on BBS proteins known to be required for ciliary trafficking, in particular on a component of the Bbsome, BBS8, and a component of the chaperonin complex, BBS6, thought to be required for Bbsome assembly [55–58].

The RPE is a ciliated epithelial monolayer essential for visual function. Insights into RPE maturation can be extrapolated to other epithelial tissues for example lung epithelial cells [19]. In mice we showed that as the RPE matures, the


a
NTC
BBS8 KD
BBS6 KD
acetyl β-catenin K49
92kDa
GAPDH
37kDa
acetyl. ß-catenin K49/GAPDH Relative density (A.U.)
b
β-catenin pT41/S45
β-catenin pT41/S45 / GAPDH Relative density (A.U.)
c
β-catenin pS33/37/T41
β-catenin pS33/37/T41 / GAPDH Relative density (A.U.)
d
acetyl β-catenin K49
Merge
NTC
NTC + Tubacin
BBS8 KD
BBS8 KD + Tubacin
BBS6 KD
BBS6 KD + Tubacin
e
Nuclear acetylated β-Catenin K49 intensity (A.U.)
Tubacin
ns
f
Ac. tubulin + PCNT2
β-catenin pS33/37/T41
g
β-catenin pS33/37/T41 positive basal bodies [%]
h
total β-catenin
i
Total ß-catenin / GAPDH Relative density (A.U.)
j
Relative Luciferase activity
Wnt3a
k
Nuclear pβ-Catenin S552 intensity (A.U.)
Non ciliated
Ciliated
Serum starved
l
Ac. tubulin
pβ-catenin S552

◂**Fig. 7** BBS-mediated regulation of ciliary disassembly components alters post-translational modification of β-catenin. Western blot analysis and quantification show reduced acetylation of β-catenin at K49 (**a**) and consequently reduced phosphorylation at p41/44 (**b**) and p33/37/41 (**c**) upon BBS8 and BBS6 knockdown (KD) in hTERT-RPE1 cells, suggesting an increase in stable and active β-catenin. Immunocytochemistry and quantification using an antibody against acetylated β-catenin K49 (green) show reduced expression in the nucleus upon BBS8 and BBS6 KD compared to non-targeting control (NTC) in hTERT-RPE1 cells (**d**). Cells were co-labeled with Arl13b (red) to confirm reduction in ciliary length. Reduced β-catenin K49 expression could be rescued by treatment with HDAC6 inhibitor tubacin ($n \geq 100$ for each group) (**d**, **e**). Immunocytochemistry using an antibody against β-catenin pS33/37/T41 (green), a target of β-catenin acetylation, shows expression at the base of the cilium and in the nucleus in NTC hTERT-RPE1 cells (**f**, **g**). Ciliary axoneme is marked by acetylated α-tubulin (Ac. tubulin, red), and the basal body by Pericentrin 2 (PCNT2, magenta). Quantification confirms reduced localization of β-catenin pS33/37/T41 at the basal body upon BBS8 and BBS6 KD (**g**). BBS8 and BBS6 KD cause less β-catenin degradation, resulting in increased levels of total β-catenin in hTERT-RPE1 cells, as quantified from Western blot (**h**, **i**). This was confirmed by a TCF/LEF luciferase activity assay that measures the transcriptional activity of β-catenin enzymatically. Luciferase activity in Wnt3a-treated non-targeting control HEK cells is upregulated compared to the untreated control. The Wnt response (luciferase activity) is significantly enhanced upon the suppression of BBS8 and BBS6 (**j**). Immunofluorescence analysis and quantification show increased stability and nuclear translocation of active β-catenin pS552 (green) in non-ciliated hTERT-RPE1 cells after BBS8 KD ($n = 263$ for NTC and 100 for BBS8 KD) (**k**, **l**). Cells were co-labeled with Ac. tubulin (green) to confirm reduction in ciliary length. Quantification of Western blot data was normalized to GAPDH levels. Bar charts in **a**–**c** show relative protein expression in arbitrary units (A.U.). Data in **a**, **b**, **c**, **i**, **j** are expressed as mean ± SD, $n = 3$ separate experiments. Data in g show mean ± SD, two independent experiments. Statistical analyses in **a**, **b**, **c**, **g**, **i** were done using the Dunnett's multiple comparison test. Data analyses in **e**, **j**, **k** were performed using Sidak's multiple comparison test. $p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; *ns* not significant. Scale bars: **a**, **e** 10 µm; **b** 2 µm; **h** 5 µm

primary cilium retracts with only a few stunted cilia being retained after birth. Cilia dysfunction in knockout mice resulted in abnormal RPE patterning in part due to defective RPE maturation (this and previous study) [19]. Retraction of the primary cilium is accompanied by expression of ciliary disassembly components suggesting that loss of the primary cilium is a tightly controlled cellular event. Interestingly, such mechanisms of ciliary disassembly are also observed in other tissues, such as in the auditory hair cells of the inner ear [6]. Here, the kinocilium plays an important role during development of the Organ of Corti but is reabsorbed by post-natal day 10 [59]. Because cilia are not retained, we suggest that they are less likely to be essential for cellular function in adult RPE, but more likely required for regulating signaling processes during development as we have recently demonstrated [19]. Since retraction of the primary cilium in the RPE is accompanied by the expression of ciliary disassembly components in vivo, we focused our attention on the molecular control of these processes.

The most common pathways that govern ciliary disassembly are through AurA, HEF1 and HDAC6 [10, 22]. Previous reports have shown that BBS proteins regulate cilia length [27] and that BBIP10 (BBS18), an additional subunit of the BBSome, interacts directly with HDAC6 [37], yet the molecular mechanisms underlying this phenomenon have not been elucidated. Here, we observed that the loss of BBS proteins resulted in increased levels of HDAC6 and that inhibition of HDAC6 resulted in a rescue of cilia length in BBS KD cells. Similarly, inhibition of AurA and HEF1 also resulted in rescue of cilia length. Therefore, we propose that BBS proteins maintain cilia length by suppressing HEF1-AurA-HDAC6-mediated disassembly (Fig. 8). Although the total levels of pAurA were not changed upon BBS KD, since inhibition of AurA activity rescued the ciliary length defect in these cells, this suggests a difference in activity.

Because Inversin (NPHP2), a key ciliary protein, influences ciliary disassembly via inhibiting AurA [35, 36] and is also a key mediator of Wnt signaling, we postulated that the role of BBS proteins in ciliary disassembly may in part be mediated via Inversin. We show that BBS proteins are required for Inversin protein expression at the base of the cilium, thereby regulating AurA phosphorylation and subsequent ciliary disassembly. AurA can also be activated by other proteins such as Pitchfork, calmodulin, trichoplein, HIF1α (Hypoxia-inducible factor 1-alpha) or Plk1(Polo-like-Kinase 1) [60]. Moreover, some kinesins such as Kif2a (Kinesin family member 2a) might also be involved as they are direct targets of AurA [61]. Therefore, BBS proteins could also be acting upon these regulators. In support of this, some of these proteins have already been shown to interact with other ciliary proteins [62, 63]. BBS proteins may also influence other ciliary length regulating components such as CPAP (Centrobin-mediated Regulation of the Centrosomal Protein 4.1-associated Protein), Nde1 (Nuclear distribution protein nudE homolog 1) and OFD1 (oral–facial–digital syndrome 1) proteins [64], which remain to be addressed.

Previous studies showed that BBS proteins interact with proteasomal subunits regulating the composition of the centrosomal proteasome [38, 65]. Consequently, loss of BBS proteins results in altered signal transduction due to defects in proteasomal clearance of key signaling proteins including β-catenin [38, 65]. Here, we show that the loss of BBS proteins decreases the stability of HEF1 and AurA proteins. A partial recovery of these proteins is observed upon inhibiting proteasomal function using MG132, suggesting that BBS proteins protect Hef1 and AurA from proteasomal degradation and thus inhibit ciliary dissasembly. Increased stability of β-catenin (discussed below) and subsequent upregulation of canonical Wnt signaling upon BBS KD may also in part be ascribable to defects in proteasomal clearance. Similarly, the cilia protein RPGRIP1L (RPGR-Interacting

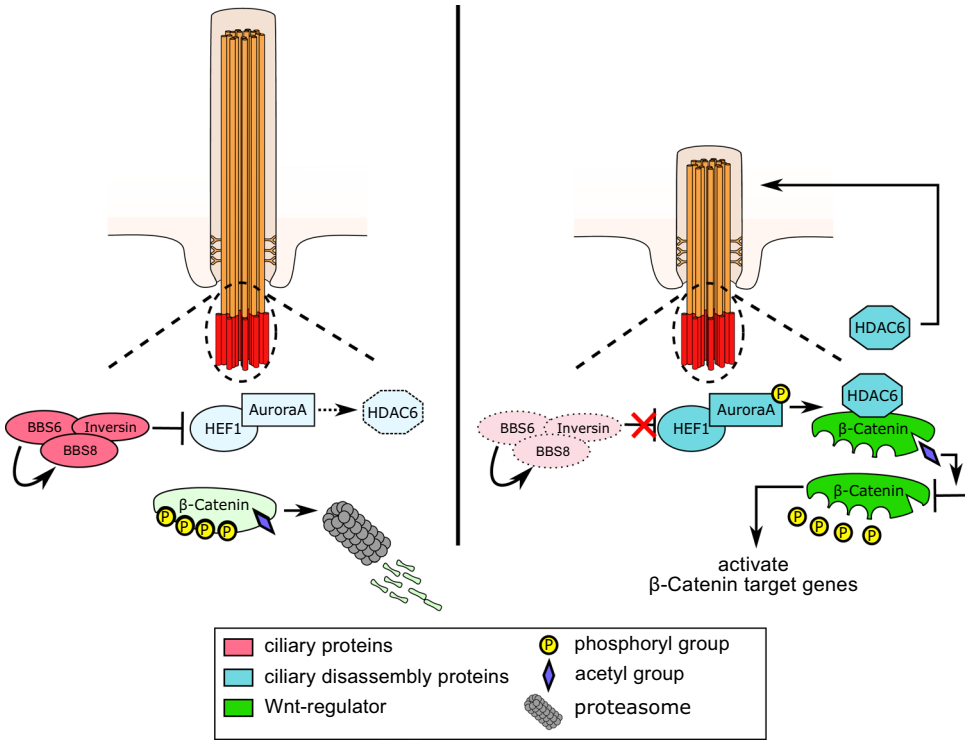


Fig. 8 Model of BBS-mediated regulation of ciliary disassembly. BBS proteins interact with Inversin and regulate its expression at the base of the cilium. Inversin inhibits HEF1/AurA, inactivating histone deacetylase HDAC6 thus preventing ciliary disassembly. As a consequence of dormant HDAC6, β-catenin remains acetylated and phosphorylated, thereby undergoing proteasomal degradation. Upon BBS suppression, Inversin expression decreases at the base of the cilium. This leads to phosphorylation and activation of AurA via HEF1, resulting in upregulation and activation of HDAC6. HDAC6 deacetylates β-catenin, hence preventing further phosphorylation and degradation. Consequently, β-catenin is stabilized and translocates to the nucleus activating canonical Wnt signaling. This also regulates ciliary length

Protein 1-Like) has been shown to protect canonical Wnt components (dishevelled) from proteasomal degradation at the basal body [53, 66]. Because HEF1 stabilization differed in BBS8 compared to BBS6 KD cells, it suggests differences in the functional role of the BBSome vs. the BBS chaperonins. One explanation for reduced protein levels of HEF1 in serum-starved BBS8 KD cells could be a feedback loop mechanism. Since it is clear from our experiments that BBS8 KD cells experience increased cilia disassembly, these cells may be trying to maintain homeostasis by mediating increased degradation of HEF1, in an effort to maintain cilia length and limit the extent of cilia disassembly. This phenomenon is intriguing, especially considering that we only see this after BBS8 KD and not after BBS6 KD, which warrants more in depth examination in the future.

In an effort to elucidate the underlying mechanisms affecting β-catenin stability upon loss of cilia function, we focused our attention on post-translational modifications (PTMs) of proteins associated with proteasomal degradation [41]. HDAC6 physically interacts and acetylates β-catenin [52] resulting in altered Wnt signaling. Several studies have demonstrated the importance of PTMs such as ubiquitination, phosphorylation, acetylation and glutamylation in various signaling processes [67]. PTMs such as phosphorylation also actively influence the process of ciliogenesis and maintenance [68]. Targeting β-catenin for degradation is a complex process involving PTMs at various sites [41]. HDAC6 deacetylation of β-catenin at K49 inhibits downstream phosphorylation. Because HDAC6 was increased in the absence of BBS proteins, we observed less acetylation at K49, which results in less β-catenin phosphorylation at T41/S45 and S33/37/T41. Thus, less β-catenin is targeted for degradation. Moreover, upon loss of BBS proteins, β-catenin is actively phosphorylated at S552, a modification that increases its stabilization and nuclear localization (our data and previous data [19]). Together, these dysregulated PTMs result in aberrant canonical-Wnt hyperactivation upon loss of BBS protein function. Although we and others [52] have observed that HDAC6 and AurA can modify β-catenin levels, very little is known about the nature of this regulation which needs further elucidation.

In conclusion, we observed transient expression of the primary cilium in the developing mouse RPE. As the RPE

matures, primary cilia retract, which are accompanied by altered expression of *Hef1*, *AurorA* and *Hdac6*, key mediators of ciliary disassembly. In control cells, BBS proteins protect against ciliary disassembly whereas the loss of BBS proteins results in altered ciliary disassembly components, including HDAC6, likely via interaction and trafficking of Inversin. This results in HDAC6-mediated downstream cross-talk between ciliary disassembly signaling and canonical-Wnt signaling, leading to PTMs resulting in canonical-Wnt hyperactivation. Taken together, we furthered our understanding into how ciliary proteins modulate cellular signaling pathways and contribute to maturation of epithelial tissues.

Materials and methods

Animals

All mouse experiments had ethical approval from appropriate governing bodies. Experiments were performed in accordance with guidelines provided by ARVO (Association for Research in Vision and Ophthalmology). Animals were housed under a 12 h light–dark cycle. The morning after mating was considered E0.5 and up to 24 h after birth was considered P0. C57BL/6 mice were used for control at embryonic (E14.5, E16.5 and E18.5), and post-natal stages (P1, P7 and adult). *Bbs6* and *Bbs8* knockout mice have been previously described [1, 69].

Cell culture

hTERT-RPE1 and HEK293T cells were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 (Thermo Fisher) or DMEM (Thermo Fisher) supplemented with heat-inactivated 10% fetal bovine serum (FBS) (LONZA), and 1% penicillin/streptomycin (P/S) (Thermo Fisher) (Referred to as complete media). Immortalized kidney medullary (KM) cells were obtained from P. Beales and cultured as previously described [27].

Antibodies and siRNAs

For immunofluorescence, antibodies used were anti-Arl13b (Rb, 1:1000; Proteintech, 17711-1-AP), anti-acetylated α-tubulin (Mm, 1:800; Sigma, T6793), anti-GT335 (Mm, 1:200; Adipogen, AG-20B-0020), anti-β-catenin (Rb, 1:150; CST, D10A8), anti- acetylated α-tubulin (Rb, 1:1000; Abcam, ab11317), anti-pericentrin2 (Gt, 1:200; SantaCruz, SC28145), and anti-ZO-1 (Mm, 1:150; Thermo Fisher, ZO1-1A12, 339188), anti-GFP (Rb, 1:5000; Clontech, 632592), anti-Aurora A (D3E4Q) (Rb, 1:100; CST, 14475), anti-phospho-Aurora A (Thr288) (C39D8) (Rb, 1:50; CST, 3079), anti-phospho-β-Catenin (Ser33/37/Thr41) (Rb, 1: 200; CST, 9561), anti-Acetyl-β-Catenin (Lys49) (D7C2) (Rb, 1: 150; CST, 9030), and anti-NPHP2 (Rb, 1:150; Abcam, ab65187). The primary antibodies were detected using Alexa Fluor 488, 555 and 568 (1:400; Molecular Probes) and CF™640R (1:400; Biotium) conjugated secondary antibodies.

For western blot, antibodies used were anti-myc-Tag (9B11) (Mm, 1:1000, CST, 2276), anti-GFP (Rb, 1:1000; Clontech, 632592), anti-Aurora A (D3E4Q) (Rb, 1:1000; CST, 14475), anti-phospho-Aurora A (Thr288) (C39D8) (Rb, 1:1000; CST, 3079), anti-β-Catenin (D10A8) (Rb, 1: 1000; CST, 8480), anti-phospho-β-Catenin (Ser33/37/Thr41) (Rb, 1: 1000; CST, 9561), anti-phospho-β-Catenin (Thr41/Ser45) (Rb, 1: 1000; CST, 9565), anti-Acetyl-β-Catenin (Lys49) (D7C2) (Rb, 1: 1000; CST, 9030), anti-HEF1/NEDD9 (2G9) (Mm, 1: 1000; CST, 4044), anti-HDAC6 (D2E5) (Rb, 1: 1000; CST 7558). Secondary antibodies used were IRDye 800 and IRDye 680 (Rb, Mm or Gt; 1:10000; Li-cor Bioscience).

For flow cytometry, a primary antibody against Aurora A (D3E4Q) (Rb, 1:100; CST, 14475) and a secondary Alexa Fluor 488-labelled anti-rabbit antibody (1: 250) were used. Propidium iodide (PI, Thermo Fisher, P3566)) at 50 µg/ml was used for cell cycle analysis.

siRNA was used in our study to knockdown (KD) *BBS6* and *BBS8* in hTERT-RPE1 cells. *BBS8* siRNA (HSC.RNAI.N198309.12; IDT), *BBS8* siRNA (L-021417-02-0005; Dharmacon), *BBS6* siRNA (L-013300-00-0005), *HEF1* siRNA (hs.Ri.NEDD9.13; IDT) and non-targeting siRNA (D-001810-10-05) were used.

Transfections and treatments: Plasmid transfections were performed using GeneTrap transfection reagent (made at NEI, NIH, Bethesda, MD, USA). In brief, 6 µl of GeneTrap transfection reagent was diluted in 90 µl of DMEM, incubated for 5 min followed by addition of 2 µg plasmid and then incubated for 20 min at room temperature (RT). The transfection mix was added dropwise to cells in a 6-well plate containing 2 ml complete media. siRNA transfections were performed in 6-well plates with Lipofectamine RNAiMax transfection reagent (Thermo Fisher; 13778150) using a reverse transfection protocol according to the manufacturer's instructions. To induce cilia formation, 24 h post-transfection cells were serum-starved with Opti-MEM (Thermo Scientific) for up to 48 h. For proteasome, AurA or HDAC6 inhibition experiments, 48 h post-transfections cells were serum-starved for 24 h followed by treatment with 10 µM MG132 (Calbiochem), 1 µM AurA Inhibitor I (Selleckchem) and 2 µM tubacin (Sigma), respectively, for the indicated time (5 h) followed by western blot analysis and immunofluorescence. DMSO was taken as vehicle control for treatments.

Tissue preparation and Immunohistochemistry

Mouse eyes were enucleated and immersed in cold phosphate-buffered saline (PBS) and kept on ice for 20 min. Eyes were then placed in 1X PBS and the cornea, lens and retina were removed. The resulting eyecups were then fixed with 4% paraformaldehyde (PFA) in PBS for 1 h at RT, then washed three times with 1X PBS and incubated with 50 mM NH_4Cl for 10 min, followed by incubation with β-Mercaptoethanol for 10 min. Eyecups were permeabilized with PBSTX (0.3% Triton-X) and blocked with blocking buffer (0.1% Ovalbumin, 0.5% Fish gelatine in PBS) at RT for 2 h followed by overnight incubation in primary antibody at 4 °C. Samples were washed three times with 1X PBS followed by incubation in secondary antibody and DAPI for 2 h at RT. Finally, samples were washed with PBS for three times before mounting on glass slides. Cells close to the optic nerve were considered near the center; cells closer to the edge of the eye cup were considered as peripheral. For isolation of pure RPE cells, eyecups were incubated with trypsin at 37 °C for 30–90 min depending on the age of tissue. RPE cells were mechanically removed from underlying choroid and isolated by hand. Choroidal and retinal contamination was checked via qPCR. Contaminated samples were removed from the analysis.

hTERT-RPE1 cells were fixed with 4% PFA for 10 min and permeabilized for 15 min with PBSTX. Immunostaining was performed as described above. KM cells were fixed with 100% methanol for 10 min on ice. Immunostaining was performed as described above.

Specimens were imaged on a Leica DM6000B microscope (Leica, Bensheim, Germany). Images were deconvoluted and co-localization profiles were generated with Leica imaging software (BlindDeblur Algorithm, one iteration step). Images were processed and cilia length measurements were performed using Fiji/ImageJ software (NIH, Bethesda, USA).

PLA assay

Direct in situ protein–protein interactions were investigated by means of a proximity ligation assay (PLA) using Duolink In Situ FarRed Kit Mouse/Rabbit (Sigma) according to the manufacturer's instruction. Cells were incubated with anti-myc and anti-Inversin primary antibodies followed by anti-rabbit PLUS and anti-mouse MINUS secondary PLA probes. The two complementary oligonucleotides were then hybridized, ligated and rolling circle amplified by the provided polymerase, resulting in fluorescence signals when the targeted proteins were closer than 40 nm. TRITC–phalloidin (Sigma) was used for visualization of cells and DAPI for nuclear staining.

Fluorescence-activated cell sorting (FACS) analyses

For FACS, RPE1 cells were stained with an antibody directed against intracellular AurA. Recovered cells were fixed using 4% PFA for 10 min and washed three times with PBS. Cells were permeabilized with 90% ice-cold methanol for 30 min and rinsed three times. Cells were blocked using 0.5% bovine serum albumin (BSA) in PBS and incubated with AurA for 1 h at RT, then washed three times with 0.5% BSA/PBS. After incubation with a secondary antibody (Alexa Fluor 488-labelled anti-rabbit) for 45 min at RT, cells were finally washed with 0.5% BSA/PBS. Cells were acquired using the Invitrogen Attune NxT Flow Cytometer (Thermo Fisher Scientific, Inc., USA), and analyzed using FlowJo (Treestar, CA, USA). For cell cycle analysis, RPE1 cells were detached and fixed with 70% ethanol for 30 min, followed by incubation with 100 µg/ml RNase A solution and 50 µg/ml propidium-iodide solution. Samples were acquired and analyzed as described above.

Pulldown and western blotting

For pulldown experiments, HEK cells were transiently co-transfected with Inversin-GFP and pCMV-BBS6/2-myc [27] or empty plasmids. 48 h post-transfections cells were lysed using RIPA buffer supplemented with EDTA-free protease inhibitor cocktail (Halt™ Protease and Phosphatase Inhibitor Cocktail (100X), Thermo Fisher). Pulldown was performed using agarose beads (GFP-Trap-A, ChromoTek) according to the manufacturer's instructions followed by western blot. Proteins were harvested in Laemmli sample buffer and separated on 8–10% SDS–polyacrylamide gel (PAGE), followed by transfer PVDF membrane (Immobilon®-FL PVDF membrane, Sigma, 05317). The blots were blocked with 5% milk or Applichem blocking buffer (0.2% AppliChem Blocking Reagent, 10 mM TrisHCl, 150 mM NaCl, 0.04% NaN3, in ddH2O; pH 7.4) and probed with antibodies as listed above. The blots were scanned for infrared fluorescence at 680 or 800 nm using the Odyssey Infrared Imaging System (Licor). Densitometry analysis was performed with Fiji/ImageJ software (NIH, Bethesda, MD, USA) and the expression levels were normalized to the input.

Quantitative real-time reverse transcriptase polymerase chain reaction

The total RNA was extracted either from hTERT-RPE1 cells, retina or RPE tissue using TRIzol reagent (Thermo Fisher, 15596026) following manufacturer's instructions. 1 µg of RNA was reverse transcribed to cDNA using GoScript reverse transcription system (Promega, A5000). cDNA was amplified on a StepOnePlus™ Real-Time PCR System

Table 1 Primers

Gene	Species	Forward	Reverse
BBS6	Human	AATGACACTGCCTGGGATG	TCGTTGTGAGTCTTGTGTCTG
BBS8	Human	ATACTCATGTGGAAGCCATCG	ATAGAAGCAACACAGCCCC
HEF1	Human	CATAACCCGCCAGATGCTAAA	CCGGGTGCTGCCTGTACT
AurA	Human	GAATGCTGTGTGTCTGTCCG	GCCTCTTCTGTATCCCAAGC
HDAC6	Human	CAACTGAGACCGTGGAGAG	CCTGTGCGAGACTGTAGC
NPHP2	Human	GCCTTCAAAATCCAAGCTGTC	CTGTTCTGCCTCTTTTCGTTTG
GAPDH	Human	GAGTCAAGGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG
Hef1	Mouse	GTACCCATCCAGATACCAAAAGG	GGAATGTCATATACCCCTTGAGG
AurA	Mouse	CACACGTACCAGGAGACTTACAGA	AGTCTTGAAATGAGGTCCCTGGCT
Hdac6	Mouse	GGAGACAACCCAGTACATGAATGAA	CGGAGGACAGAGCCTGTAG
Arl13b	Mouse	AGCGGATGTGATTGAGTGTC	ACAAGGTTCGATCTGACACAG
Ift20	Mouse	AAGGAACCAAAGCATCAAGAATTAG	AG ATGTCATCAGGCAGCTTGAC
Prph2	Mouse	TCTCCTCCAAGGAGGTCAAAG	GAGTCCGGCAGTGATGCTCAC
Rpe65	Mouse	ACTTCCCCTTTCAATCTCTTCC	TTTTAACTTCTTCCCAATTCTCACG
Cdh5	Mouse	ACACCTTCACCATTGAGACAG	CTGCTCAGGTATTCGTATCGG
Gapdh	Mouse	CGACTTCAACAGCAACTCCCACTCTTCC	TGGGTGGTCCAGGGTTTCTTACTCCTT

(Applied Biosystems, 4376600) using SYBR Green (Thermo Fisher; Platinum™ SYBR™ Green qPCR SuperMix-UDG, 11733046) according to the manufacturer's recommendation. The following cycling conditions were used: 95 °C for 10 min followed by 40 cycles of 95 for 15 s, 60 for 1 min. Specificity of the amplified product was determined by melt curve analysis. Relative target gene expression was normalized to GAPDH and analyzed by comparative Ct or $2^{\Delta\Delta CT}$ method [70, 71]. For a list of primers used, see Table 1.

Electron microscopy

Electron microscopy was performed as previously described [72].

Luciferase assay

To monitor the activity of the Wnt/β-catenin signaling pathway characterized by TCF/LEF-dependent target gene transcription, reporter gene assays were performed with the Dual-Glo® Luciferase Assay System (Promega) in a 96-well plate. BBS6 and BBS8 siRNA-mediated KD in HEK293T cells were performed using Lipofectamine RNAiMax transfection reagent (Thermo Fisher) using a reverse transfection protocol according to the manufacturer's instructions. After 24 h cells were transiently transfected with plasmids by X-tremeGENE™ 9 DNA Transfection Reagent (Sigma) according to the manufacturer's protocol. Plasmids used were pRL-TK (Renilla luciferase, 1 ng), TopFlash (5 ng) and the total amount of transfected DNA was equalized to 80 ng by addition of pcDNA3. 24 h post-transfections cells were stimulated with Wnt3a conditioned media and luciferase activity was measured after 24 h in a Tecan Infinite M200 Pro plate reader. Firefly luciferase activity was normalized to Renilla luciferase activity in each well.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA). For multigroup comparisons, ANOVA followed by Dunnett's multiple comparison test, Turkeys multiple comparison test and Sidak's multiple comparison test was performed depending of the data to be compared. Differences between two groups were compared using a nonparametric Mann–Whitney *U* test. *p* value of 0.05 and below was considered statistically significant. Statistical tests and number of repetitions are described in the legends. Box plots show median (middle line), edge of boxes is top and bottom quartiles (25–75%), and whiskers represent the ranges for the upper 25% and the bottom 25% of data values. Outliers were excluded using the ROUT method (GraphPad Prism). Bar plots show mean ± standard deviation (SD).

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Author contributions SRP and HLM-S were responsible for conception and experimental design. SRP, VK, LB, A-KV, SS, LRO-C and

HLM-S performed experiments. SRP and VK generated figures. SRP and HLM-S co-wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declares that they have no conflict of interest.

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Manuscript I

The actin-bundling protein Fascin-1 modulates ciliary signalling.

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Abstract

Primary cilia are microtubule-based cell organelles important for cellular communication. Since they are involved in the regulation of numerous signalling pathways, defects in cilia development or function are associated with genetic disorders, collectively called ciliopathies. Besides their ciliary functions, recent research has shown that several ciliary proteins are involved in the coordination of the actin cytoskeleton. However, the interconnection of ciliary and actin phenotypes is not fully understood. Here we show that the protein BBS6/MKKS, associated with the ciliopathy Bardet-Biedl syndrome, cooperates with the actin-bundling protein Fascin-1 in regulating filopodia and ciliary signalling. We found that loss of *Bbs6* affects filopodia length potentially via interaction with Fascin-1. Conversely, loss of Fascin-1 leads to a ciliary phenotype, subsequently affecting ciliary Wnt signalling, possibly in collaboration with Bbs6. Our data shed light on how ciliary proteins are involved in actin regulations and provide new insight into the involvement of the actin regulator Fascin-1 in ciliogenesis and cilia-associated signalling. Expanding our knowledge of the complex regulations between primary cilia and actin dynamics is important to understand the pathogenic consequences of ciliopathies. With Fascin-1, we have further expanded the repertoire of potential ciliopathy genes that should be screened for in undiagnosed ciliopathy patients.

Introduction

Primary cilia are microtubule-based sensory cell protrusions vital for cell homeostasis and tissue development. They act as sensory antennae, receiving and transducing cues related to cellular signalling pathways such as sonic hedgehog (Shh), platelet-derived growth factor (PDGF) or Wnt (Goetz and Anderson, 2010; Wallingford and Mitchell, 2011; May-Simera and Kelley, 2012; Lee, 2020). Thus, defects in primary cilia or ciliary proteins are known to be associated with a group of genetic disorders, so-called ciliopathies (Reiter and Leroux, 2017; Chen et al., 2021). Although primary cilia are predominantly microtubule-based organelles which coordinate the cellular microtubule network, ciliary defects also disrupt regulation of the actin cytoskeleton (Brücker et al., 2020; Smith et al., 2020). Conversely, actin polymerisation is a negative regulator of ciliogenesis (Bershteyn et al., 2010; Kim et al., 2010, 2015; Liang et al., 2016). The interconnected phenotype between primary cilia and actin dynamics is complex and not fully understood, which is why any new insights into these processes would have wide reaching consequences.

Numerous ciliary proteins have already been shown to coordinate the actin cytoskeleton, emphasising the interplay between cilia and actin (Yin et al., 2009; Kim et al., 2010; May-Simera et al., 2016). An important subset of cilia proteins involved in actin dynamics are the BBS proteins, defects in which are associated with the archetypical ciliopathy Bardet-Biedl syndrome (BBS). Besides their classically defined function in cilia development, maintenance and trafficking (Wei et al., 2012; Nozaki et al., 2019; Patnaik et al., 2019), BBS proteins have been shown to be associated with downstream actin perturbations (Novas et al., 2015). Loss of BBS4, 6, 8 or 15, which exert different ciliary functions, result in defective actin-based cell migration and a disorganisation of the actin cytoskeleton (Cui et al., 2013; Hernandez-Hernandez et al., 2013). This is associated with upregulation of downstream RhoA signalling resulting in increased numbers of focal adhesions. In zebrafish, bbs8 was further found to be required for neural crest cell migration and migration of fibroblasts, and its loss was accompanied by a lack of polymerisation of the actin cytoskeleton and disorganised microfilaments (Tobin et al., 2008). However, the direct link between ciliary BBS proteins and the actin cytoskeleton is still unclear.

Many actin phenotypes caused by ciliary defects thus far could be ascribed to aberrant non-canonical Wnt signalling (planar cell polarity, PCP), a pathway known to affect downstream actin networks (Gerdes et al., 2007; Corbit et al., 2008; May-Simera et al., 2010, 2015; Cui et al., 2013; McMurray et al., 2013; Balmer et al., 2015; Wang et al., 2017). Upon activation of this pathway by Wnt ligands binding to the Frizzled receptor, the ciliary PCP protein Inversin recruits Dishevelled to the plasma membrane (Simons et al., 2005). Dishevelled in turn binds to formins and small GTPases, subsequently activating downstream actin regulators such as RhoA, Rock and Jnk (Habas et al., 2001; Liu et al., 2008). Thus, Inversin acts as a key player in the switch from canonical to non-canonical Wnt signalling, inhibiting canonical Wnt and promoting directional cell migration via regulation of Rho GTPases and the downstream actin network (Simons

et al., 2005; Lienkamp et al., 2012; Veland et al., 2013; Werner et al., 2013). PCP signalling also results in the development of actin-based signalling filopodia which further distribute the Wnt signal to recipient cells (Stanganello et al., 2015; Mattes et al., 2018; Rosenbauer et al., 2020). Recent data indicate that BBS2, BBS6 and BBS8 interact with Inversin, facilitating its transport to the base of the cilium and enabling its function in non-canonical Wnt signalling (May-Simera et al., 2018; Patnaik et al., 2019). Consistently, knockout of bbs6 and bbs8 in zebrafish lead to enhanced canonical Wnt signalling and a PCP phenotype, supporting the cooperation between Inversin and BBS proteins (Ross et al., 2005; Gerdes et al., 2007; May-Simera et al., 2010, 2015).

Besides the reciprocity between ciliary proteins and Wnt signalling in regulating actin dynamics, it is plausible that there is a more direct connection between cilia and the actin cytoskeleton. Numerous proteins have been associated in directly regulating the actin cytoskeleton, enabling cell migration, trafficking and morphology. Since primary cilia are microtubule-based organelles, actin-binding proteins that also affect the microtubule network are of particular interest. Prominent examples for this include MACF1, the microtubule-actin crosslinking factor, the inverted formin 1 (FHDC1), and Fascin-1, an actin-bundling protein important for filopodia formation (Wu et al., 2008; Young et al., 2008; Thurston et al., 2012; Antonellis et al., 2014; Villari et al., 2015). For MACF1 and FHDC1, a role in ciliogenesis has already been described (May-Simera et al., 2016; Copeland et al., 2018); however, a functional link between Fascin-1 and ciliary proteins has not been investigated.

In the current work, we shed light on the functional regulation between ciliary proteins and actin. We show that *Bbs6* deficient cells exhibit a filopodia phenotype, correlating with BBS6 building a complex with the filopodia regulator Fascin-1. Interestingly, knockdown of *Fascin-1* affected cilia number, suggesting a role of Fascin-1 in initiating ciliogenesis. Furthermore, we found Fascin-1 that cooperates with Bbs6 in regulating ciliary PCP/Wnt signalling via downstream Wnt targets such as Cyclin D1. Taken together, our results demonstrate a role for Fascin-1 in bridging the regulation of primary cilia and actin networks, connecting phenotypes in both ciliogenesis and actin via coordination of signalling pathways such as Wnt.

Results

Bbs6 regulates filopodia stability via interaction with Fascin-1.

Loss of ciliary proteins Bbs6 or Bbs8 was previously shown to be associated with defects in cell migration and disrupted actin networks (Tobin et al., 2008; Hernandez-Hernandez et al., 2013). To further elucidate this association, we analysed filopodia in mouse embryonic fibroblasts (MEFs). Filopodia are actin-based cell protrusions that sense the environment and are thus important for efficient cell migration (Amarachintha et al., 2015). Cells co-expressing mRFP-tagged Lifeact as a marker for the actin cytoskeleton and the EGFP-tagged filopodia regulator Fascin-1 were used to perform live cell imaging to visualise filopodia (Supp. Movies). Analysis of the resulting movies

revealed that *Bbs6*$^{-/-}$ MEFs assembled shorter filopodia compared to wildtype cells (Fig. 1 A, B; Supp. Movie 1, 2), although localisation of Fascin-1 to filopodia appeared to be similar in fixed cells (Fig. 1 A, C), potentially due to loss of filopodia stability during fixation. This filopodia phenotype is supported by previous studies where a disruption of filopodia was assumed to be the cause of migration defects observed in *Bbs6* knockout kidney medullary cells (Hernandez-Hernandez et al., 2013). Despite previous findings that loss of *Bbs8* hinders cell migration (Tobin et al., 2008; Hernandez-Hernandez et al., 2013), *Bbs8*$^{-/-}$ MEFs did not display a phenotype in filopodia length (Supp. Fig. 1 A, B; Supp. Video 3, 4), suggesting Bbs8 is not involved in this aspect of cell environmental sensing.

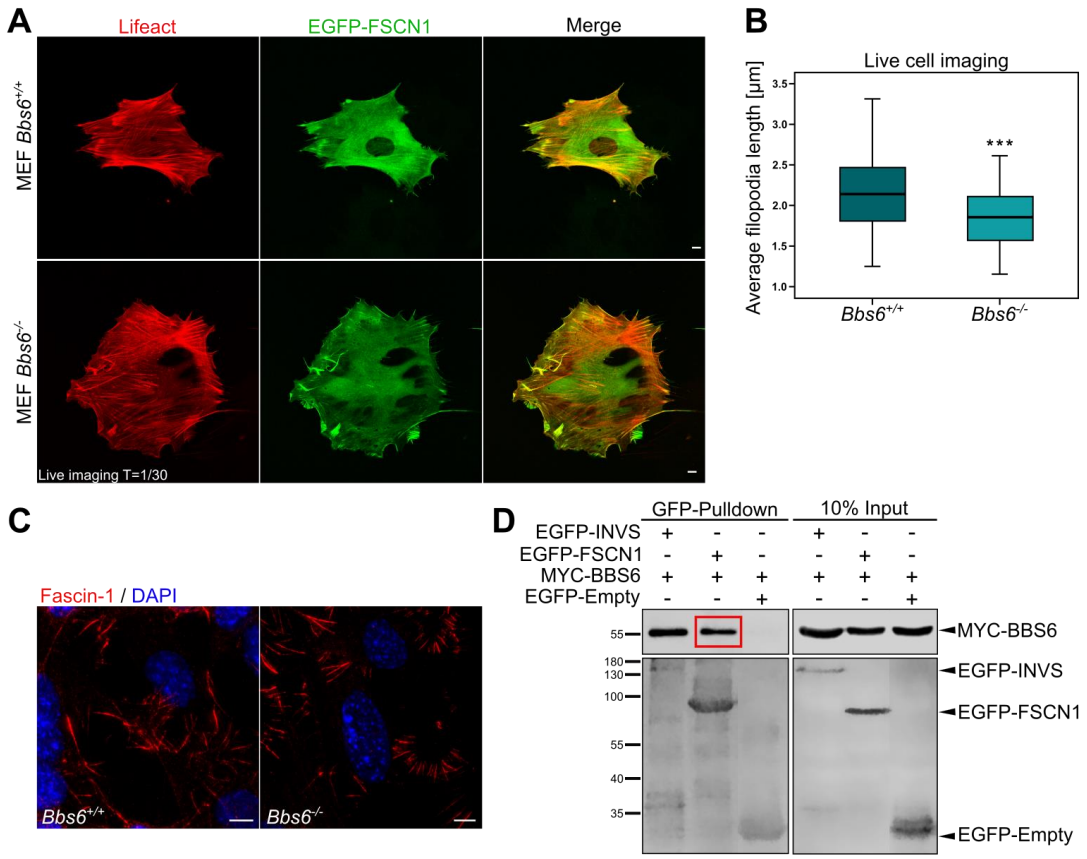

Fig. 1: Loss of the ciliary protein Bbs6 disrupts filopodia dynamics via interaction with Fascin-1. **A** Live cell imaging of Lifeact (actin cytoskeleton) and EGFP-FSCN1 (filopodia) in *Bbs6* wildtype (*Bbs6*$^{+/+}$) and knockout (*Bbs6*$^{-/-}$) mouse embryonic fibroblasts (MEFs), 48 h after transfection. Images represent one timepoint out of 30, videos are shown in supplementary data. **B** Average filopodia length via FiloQuant analysis shows significantly shorter filopodia upon *Bbs6* knockout (Mann-Whitney-U test, p=0.000001). N(*Bbs6*$^{+/+}$)=93, N(*Bbs6*$^{-/-}$)=70. **C** *Bbs6* MEFs fixed with methanol and stained with Fascin1 (red) for filopodia visualisation. Fascin-1 localises to filopodia in *Bbs6* wildtype and knockout MEFs, showing no defect in localisation. **D** Interaction study between Fascin-1 and Bbs6. GFP pulldowns were performed 48 h after overexpression of EGFP-FSCN1 and MYC-BBS6 in HEK293T cells. The interaction between EGFP-INVS (Inversin) and MYC-BBS6 was used as positive control, EGFP-empty served as negative control. Red box shows formation of a complex between EGFP-FSCN1 and MYC-BBS6. Blots represent a cropped version of Supp. Fig. 2C. Scale bars: 10µm. *p<0.05, **p<0.01, ***p<0.001. Experiments were repeated at least 3 times.

Since Fascin-1 is the key actin-bundling protein organising F-actin structures in filopodia (Kureishy et al., 2002; Vignjevic et al., 2006; Pfisterer et al., 2020), we adopted a candidate approach to determine whether the ciliary proteins Bbs6 and Bbs8 form a complex with Fascin-1. EGFP-FSCN1, MYC-BBS6, MYC-BBS8 or empty vector controls were expressed in HEK293T cells and GFP pulldown experiments performed to assess complex formation. The previously described interaction between BBS6 and Inversin/INVS was used as a positive control (Patnaik et al., 2019). EGFP-FSCN1 pulled down MYC-BBS6, but not MYC-BBS8 (Fig. 1 D; Supp. Fig. 1 C). Taken together, these data indicate a regulation of filopodia via Bbs6, but not Bbs8, possibly via association with Fascin-1.

Functional interaction between Bbs6 and Fascin-1 in cell migration is independent of transcriptional regulation.

To understand whether Bbs6 and Fascin-1 may cooperate by influencing stability of expression of key regulatory proteins, we sought to investigate how these proteins influence one another. For this, we analysed the expression levels of actin and ciliary components (Arl13b and poly-glutamylated tubulin) upon loss or knockdown of *Bbs6* and *Fascin-1*.

Analysis of *Bbs6* knockout cells showed no change in levels of Fascin-1, actin and Arl13b proteins, but a significant reduction in poly-glutamylated tubulin indicating a regulation of Bbs6 in the posttranslational modification of tubulin (Fig. 2 A, B). Knockdown of *Fascin-1* in MEFs was performed using siRNA and validated via RT-qPCR, western blots and immunofluorescence (Supp. Fig. 2). No changes in expression levels of any of the target proteins were detected upon knockdown of *Fascin-1,* noting the lack of a reliable antibody for Bbs6 precluded us from analysing levels of this protein. Loss of *Bbs6* or *Fascin-1* did not affect mRNA levels of *beta-actin*, *Arl13b* or *Bbs6* (Fig. 2 C). Taken together, these results show that, with the exception of poly-glutamylated tubulin, the transcription and translation of these targets are not dependent on Bbs6 or Fascin-1.

It was previously reported that BBS proteins play a role in the ubiquitination of signalling receptors and their retention inside the primary cilium (Xu et al., 2015; Shinde et al., 2020). Fascin-1 is monoubiquitinated at Lys247 and Lys250 within the second actin-binding site (Lin et al., 2016). Although we did not see a change in total protein level of Fascin-1 upon knockout of *Bbs6* (Fig. 2 A, B), we sought to determine whether Bbs6 is involved in the ubiquitin-dependent degradation of Fascin-1. We immunoprecipitated endogenous Fascin-1 from *Bbs6* wildtype and knockout cells treated with and without the proteasome inhibitor MG132 to prevent degradation of ubiquitinated Fascin-1 protein. After probing the western blots with an antibody against Ubiquitin, we did not see any increase or change in the amount of ubiquitinated Fascin-1 between wildtype and knockout samples (Supp. Fig. 3), indicating that Bbs6 has no effect on ubiquitin-dependent proteasomal degradation of Fascin-1. These findings

indicate that functional crosstalk between Bbs6 and Fascin-1 does not occur through control of protein stability or expression of each protein.

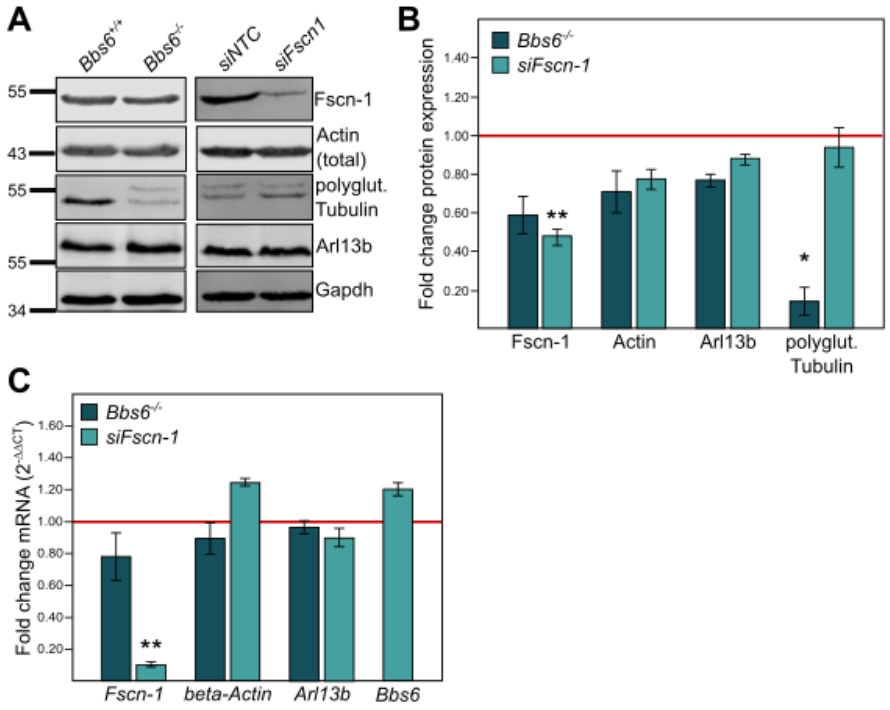

Fig. 2: Transcriptional regulation of Bbs6 and Fascin-1.
A Protein levels of Fascin-1, Actin, poly-glutamylated tubulin (detected via Gt335) and Arl13b in MEFs shown by western blots. **B** Analysis of the protein expression levels in western blots (AUC) in *Bbs6*$^{-/-}$ knockout and *Fascin* knockdown MEFs, normalised to Gapdh and in comparison to the WT/non-targeting control (*siNTC)* sample (red line). No significant differences in the expression of all proteins could be detected, except for poly-glutamylated tubulin, which was significantly downregulated in *Bbs6*$^{-/-}$ MEFs (p=0.02) and *Fascin-1*, whose expression is downregulated upon its knockdown. **C** mRNA levels of *Fascin-1*, *beta-actin*, *Arl13b* and *Bbs6* in MEFs measured via RT-qPCR. No significant differences in the mRNA levels of all genes, except for *Fascin-1*, whose expression is downregulated upon its knockdown. Normalisation to the wildtype/*siNTC* samples (red line). Student's t-test: n.s. p>0.05, *p<0.05, **p<0.01, ***p<0.001. Experimental procedures were repeated at least 3 times.

Loss of Fascin-1 is associated with a ciliary phenotype in mouse embryonic fibroblasts.

The observed complex between Fascin-1 and Bbs6 and the filopodia phenotype in *Bbs6* depleted cells suggests that these proteins cooperate in cellular processes. Although the primary cilium is predominantly a microtubule-based structure, actin related proteins also play an important role in ciliogenesis (Bershteyn et al., 2010; Kim et al., 2010, 2015; Pitaval et al., 2010; Liang et al., 2016). To investigate whether this might be also true for Fascin-1, we quantified number and length of primary cilia after *Fascin-1* knockdown.

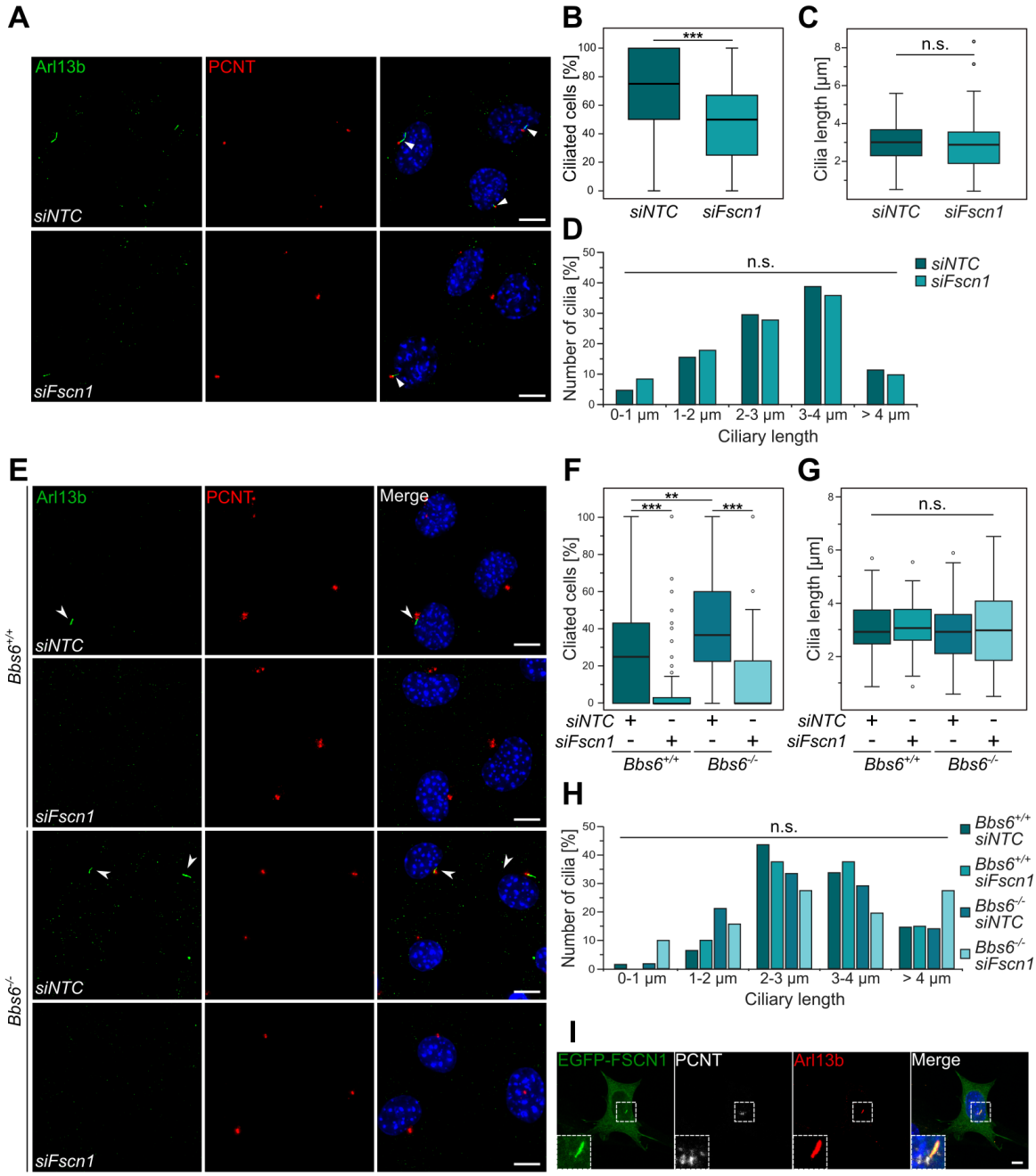

Fig. 3: Loss of Fascin-1 causes a ciliary phenotype.

A Loss of *Fascin-1* via siRNA mediated knockdown in MEFs (*siFscn1*) led to significantly reduced numbers of ciliated cells compared to transfection with non-targeting control siRNAs (*siNTC*). Transfected MEFs were cultured in serum-depleted medium for 24 h to induce ciliation before fixation with 4% PFA. Visualisation of primary cilia (arrow heads) with the ciliary membrane marker Arl13b (green) and the basal body protein PCNT (red). Scale bar: 10µm. **B** Quantification of ciliated cells upon knockdown of *Fascin-1*. Knockdown of *Fascin-1* results in significantly reduced cilia numbers (Mann-Whitney-U test, p=0.000037), N(*siNTC*)=438, N(*siFscn1*)=395. **C** Analysis of ciliary length of *siNTC* and *siFscn1* did not reveal significant differences (Mann-Whitney-U test, p=0.10461), N(*siNTC*)=315, N(*siFscn1*)=212. **D** Numbers of cilia according to their subclass of length show no significant differences between *siNTC* and *siFscn1*. Subclasses were divided between 0-1µm, 1-2µm, 2-3µm, 3-4µm and above 4µm and numbers of each cilia class are given in percentage. **E** *Bbs6* MEFs transfected with *siNTC* and *siFscn1* were cultured in serum-depleted medium before fixation with 4% PFA. Visualisation of primary cilia (arrow heads) with the ciliary membrane marker Arl13b (green) and the basal body

protein PCNT (red). Scale bars: 10µm. **F** Quantification of ciliated cells upon knockdown of *Fascin-1* (*siFscn1*) in *Bbs6* wildtype and knockout cells reveals a significant upregulation in cilia numbers upon loss of *Bbs6* and downregulation upon loss of *Fascin-1*. Double loss of *Bbs6* and *Fascin-1* rescues cilia numbers again, showing no significant difference in comparison to *Bbs6*$^{+/+}$ *siNTC*. N(*Bbs6*$^{+/+}$*,siNTC*)=462, N(*Bbs6*$^{+/+}$*,siFscn1*)=458, N(*Bbs6*$^{-/-}$*,siNTC*)=393, N(*Bbs6*$^{-/-}$*,siFscn1*)=363. **G** Analysis of ciliary length between *siNTC* and *siFscn1* in both *Bbs6* wildtype and knockout MEFs showing no significant differences between all samples. N(*Bbs6*$^{+/+}$*,siNTC*)=123, N(*Bbs6*$^{+/+}$*,siFscn1*)=42, N(*Bbs6*$^{-/-}$*,siNTC*)=156, N(*Bbs6*$^{-/-}$*,siFscn1*)=51. **H** Numbers of cilia according to their subclass of length reveals no significant differences in length subclasses upon loss of *Bbs6* and/or *Fascin-1*. Subclasses were divided between 0-1µm, 1-2µm, 2-3µm, 3-4µm and above 4µm and numbers of each class are given in percentage. **I** Visualisation of overexpressed EGFP-FSCN1 in serum-depleted MEFs. Costaining of Arl13b and PCNT as cilia proteins revealed localisation of EGFP-FSCN1 to cilia. Scale bar: 8µm. Mann-Whitney-U test: n.s. $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$. Data represent at least three independent experiments.

Loss of *Fascin-1* in MEFs led to significantly reduced numbers of ciliated cells (~50%) compared to transfection with non-targeting control siRNAs (~80%) (Fig. 3 A, B). We also quantified primary cilia length to analyse whether these ciliation defects are due to a deficiency in the process of cilia initiation, assembly, or disassembly. Despite fewer cilia, no changes in cilia length were seen following *Fascin-1* depletion (Fig. 3 C, D), suggesting the ciliation defect is most likely a consequence of failed initiation.

Loss of *Bbs6* has been shown to increase ciliation in kidney medullary cells (Volz et al., 2021). To further analyse the interplay between Bbs6 and Fascin-1 in ciliogenesis, we analysed whether knockdown of *Fascin-1* in *Bbs6* depleted cells could rescue their ciliary phenotype. As expected, knockout of *Bbs6* lead to a significant increase in cilia number (Fig. 3 E, F), although length was not affected (Fig. 3 G-H). *Fascin-1* depletion in *Bbs6* knockout cells resulted in a reduction in the enhanced cilia numbers, showing a rescue in the ciliation phenotype (Fig. 3 F). These results show that Fascin-1 is required for ciliogenesis in MEFs and potentially acts antagonistically to Bbs6.

Fascin-1 has been shown to localise to primary cilia of murine fibroblasts, where it was suggested to assist actin based ciliary decapitation (Phua et al., 2017). To verify this Fascin-1 localisation, we overexpressed EGFP-tagged Fascin-1 in MEFs and performed fluorescence microscopy. A distinct localisation of EGFP-FSCN1 at both the basal body and along the ciliary axoneme was observed in a small proportion of cells (Fig. 3 I), suggesting this recruitment is either transient or linked to a specific state of ciliogenesis.

The actin and microtubule binding properties of Fascin-1 can be influenced via the manipulation of S39 and S274 phosphorylation sites within the two actin-binding domains (Villari et al., 2015). We therefore tested whether overexpression of the phospho-mimetic construct with decreased actin-binding capacity (S274D) showed stronger localisation to primary cilia as it binds to microtubules more efficiently. Expression of this mutant did not increase ciliary localisation of Fascin-1, and nor did the overexpression of other mutant forms of Fascin-1 (S39A, S39D, S274A) that are known to influence cytoskeletal binding (Supp. Fig. 4). Taken together, we identified

Fascin-1 as a positive regulator of ciliogenesis that can transiently be recruited to the cilium.

Fascin-1 is involved in the regulation of ciliary Wnt signalling.

Since loss of Fascin-1 results in a reduction in ciliated cells, we sought to determine further downstream effects. Primary cilia regulate several signalling pathways, including Wnt signalling which can modulate actin network assembly (Fig. 4 A) (Gerdes et al., 2007; Corbit et al., 2008; May-Simera et al., 2010, 2015; Cui et al., 2013; McMurray et al., 2013; Balmer et al., 2015; Wang et al., 2017). BBS6 and Inversin interact and regulate the switch from canonical to non-canonical Wnt signalling (Simons et al., 2005; Gerdes et al., 2007; Patnaik et al., 2019). Since we identified Fascin-1 in a complex with BBS6, we hypothesised that it might also interact with Inversin. We overexpressed mRFP-tagged FSCN1 and EGFP-INVS or empty vector controls and performed GFP pulldown experiments to assess complex formation. The interaction between MYC-BBS6 and EGFP-INVS was used as a positive control (Patnaik et al., 2019). EGFP-INVS pulled down mRFP-FSCN1, indicating an interaction between both proteins (Fig. 4 B).

These data suggest a function for Fascin-1 in cilia-related Wnt signalling. For this, we examined mRNA levels of key Wnt signalling proteins after knockdown of *Fascin-1* in comparison to *Bbs6* knockout. Knockdown of *Fascin-1* in MEFs led to a significant increase in mRNA expression of *Cyclin D1*, a downstream target of canonical Wnt, although other Wnt effectors were not affected (Fig. 4 C). This effect was mirrored by loss of *Bbs6*, also significantly enhancing *Cyclin D1* mRNA expression levels in addition to other Wnt pathway components *Lrp5/6*, *Dvl2, Inversin* and *Axin2* (Fig. 4 B). Since loss of *Bbs6* in known to activate canonical Wnt signalling (Gerdes et al., 2007; Patnaik et al., 2019; Volz et al., 2021), it is plausible that this is facilitated via a transcriptional upregulation of *Cyclin D1* and *Lrp6* and downregulation of *Axin2*. Dvl2 is known to interact with Inversin in mediating non-canonical Wnt. Thus, the transcriptional upregulation of these genes might be a mechanism to compensate the hyperactivation of canonical Wnt due to loss of *Bbs6*.

Upregulation of Cyclin D1 upon loss of *Fascin-1* was confirmed via immunocytochemistry. The intensity of Cyclin D1 inside the nucleus was significantly enhanced upon knockdown of Fascin-1 (Fig. 4 D, E), suggesting an activation of canonical Wnt signalling (Shtutman et al., 1999; Tetsu and McCormick, 1999). Since primary cilia were shown to modulate Wnt signalling (Gerdes et al., 2007; Corbit et al., 2008), these data indicate the upregulation of canonical Wnt signalling being a consequence of the ciliary phenotype.

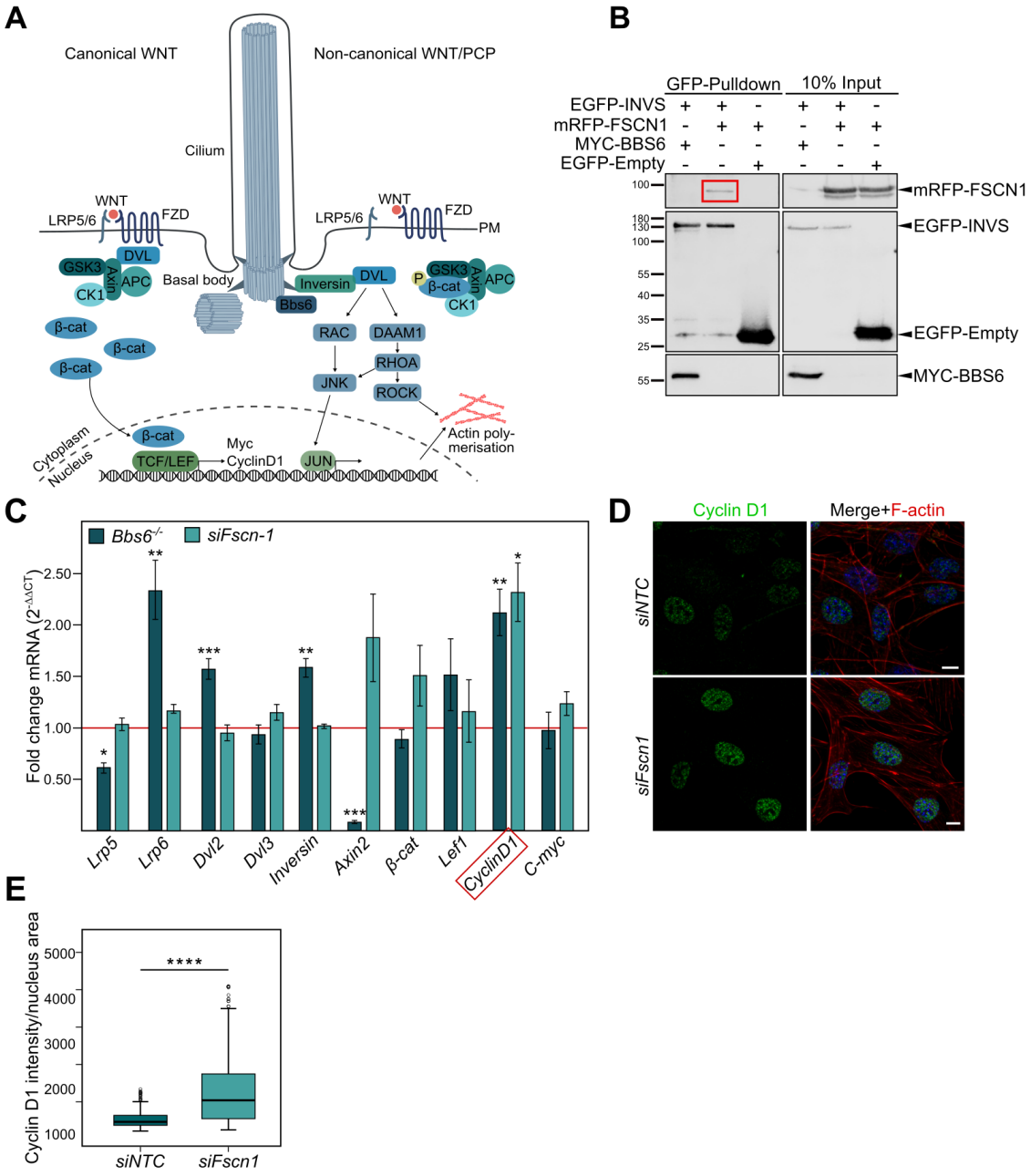

Fig. 4: Fascin-1 regulates ciliary Wnt signalling.
A Graphical representation of canonical and non-canonical (PCP) Wnt signalling. Upon activation of canonical Wnt via binding of the Wnt ligand to a coreceptor complex consisting of LRP5/6 and Frizzled (FZD), Dishevelled (DVL) inactivates the beta-catenin degradation complex (Axin, GSK3, APC, CK1). Beta-catenin (β-cat) accumulates and enters the nucleus where it acts as coactivator of transcription factor TCF/LEF that activate the transcription of Wnt target genes such as Cyclin D1 and Myc. During non-canonical Wnt, Dishevelled is translocated via Inversin, so that beta-catenin gets degraded. Dishevelled activates downstream signalling cascades activating actin networks via regulation of Rac, Daam and Rho GTPases. **B** Interaction study between Fascin-1 and Inversin/INVS. GFP pulldowns were performed 48 h after overexpression of mRFP-FSCN1 and EGFP-INVS in HEK293T cells. The interaction between EGFP-INVS and MYC-BBS6 was used as positive control, EGFP-empty served as negative control. Red box shows formation of a complex between mRFP-FSCN1 and EGFP-INVS. **C** mRNA levels of Wnt signalling genes upon knockdown of *Fascin-1* or knockout of *Bbs6* in MEFs measured via RT-qPCR. Data shown as fold changes in comparison to the WT/*siNTC* control (red line).

Differential expression of Lrp5/6, Dvl2, Inversin, Axin2 and Cyclin D1 in *Bbs6*$^{-/-}$ MEFs, whereas only Cyclin D1 was significantly upregulated upon loss of *Fascin-1* (red box). Student's t-test. **D** Visualisation of Cyclin D1 (green) inside the nucleus of MEFs transfected with *siNTC* and *siFscn1* 48 h prior fixation with 4% PFA shows a higher signal of Cyclin D1 upon loss of *Fascin-1*. Scale bar: 10µm. **E** Quantification of the fluorescence intensity of Cyclin D1 inside the nucleus of MEFs measured with Fiji in relation to the nucleus area. Nuclear Cyclin D1 is significantly enhanced upon knockdown of *Fascin-1* in MEFs ($p=8.98*10^{-26}$). N(*siNTC*)=297, N(*siFscn1*)=282. Mann-Whitney-U test. n.s. p>0.05, *p<0.05, **p<0.01, ***p<0.001. Data represent at least three independent experiments.

Fascin-1 cooperates with cilia proteins in modulating Wnt signalling.

Given that loss of both *Bbs6* and *Fascin-1* led to increased Cyclin D1 levels, we next tested whether Bbs6 and Fascin acted synergistically in this pathway. We quantified Cyclin D1 within the nucleus of *Bbs6*$^{-/-}$ MEFs after knockdown of *Fascin-1* (Fig. 5 A-C). In *Bbs6* wildtype cells, knockdown of *Fascin-1* led to an increase of nuclear Cyclin D1 levels, a similar phenotype to that seen in *Bbs6*$^{-/-}$ cells (Fig. 5 A, B) (Volz et al., 2021). Since the intensity of Cyclin D1 in *Bbs6*$^{-/-}$ cells was so high, microscope illumination settings had to be reduced to avoid saturation and enable subsequent analysis (Fig. 5 A, lower panel, and C). Interestingly, this revealed a significant decrease of nuclear Cyclin D1 in *Bbs6*$^{-/-}$ cells upon *Fascin-1* knockdown.

Besides being regulated via Wnt signalling, the nuclear localisation of Cyclin D1 is also controlled via the cell cycle. Although it was previously shown that loss of *Bbs6* is not associated with major changes in G1-S phase transition, which regulates Cyclin D1 translocation (Kim et al., 2005; Patnaik et al., 2019), we examined a second Wnt signalling target to determine whether this correlated with Cyclin D1 changes. One of the canonical Wnt effectors, beta-catenin, is acetylated at Lys49 via CREB-binding protein (CBP), regulating its transcriptional activity in a promoter specific fashion (Wolf et al., 2002). In wildtype MEFs, localisation of acetylated beta-catenin was restricted to the nucleus (Fig. 5 D), making it a suitable marker for Wnt activity.

Significantly enhanced nuclear levels of acetylated beta-catenin were seen in both *Fascin-1* knockdown and *Bbs6* knockout cells (Fig. 5 D, E). However, additional knockdown of *Fascin-1* did not reverse this phenotype as seen before for Cyclin D1. This suggests that Bbs6 and Fascin-1 do not completely overlap in terms of their functional Wnt effect.

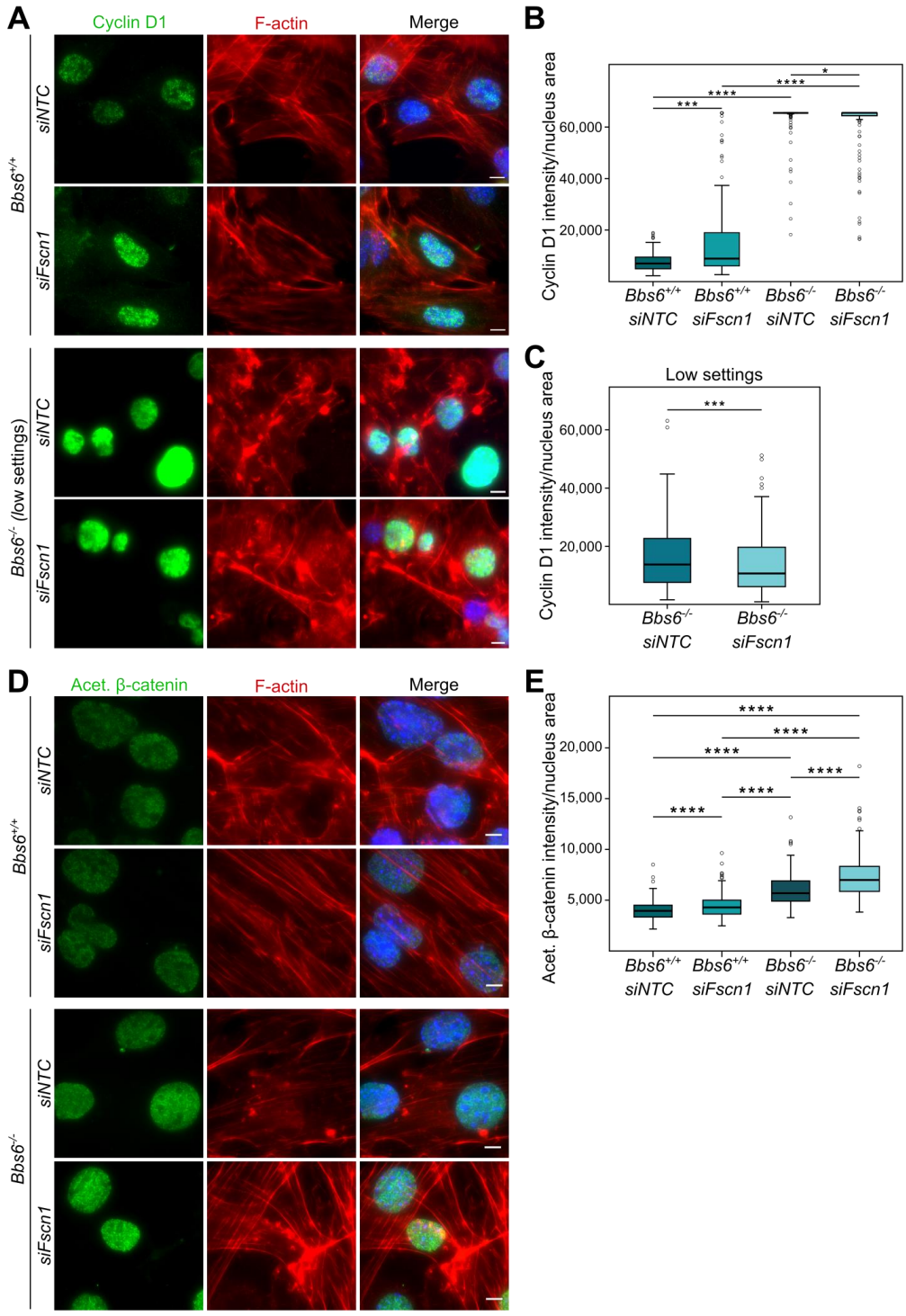

Fig. 5: Fascin-1 regulates ciliary Wnt signalling in cooperation with Bbs6.
A-C Quantification of nuclear Cyclin D1 after loss of *Bbs6* and *Fascin-1*. **A** Visualisation of Cyclin D1 inside the nucleus of *Bbs6* MEFs transfected with *siNTC* and *siFscn1* 48 h prior fixation with 4% PFA. For Cyclin D1 in *Bbs6*$^{-/-}$ MEFs (lower panel), microscope illumination settings were reduced to avoid saturation and enable subsequent analysis. Scale bars: 10µm. **B** Quantification of the fluorescence intensity of Cyclin D1 inside the nucleus of *Bbs6* MEFs transfected with *siNTC* or *siFscn1* measured with Fiji in relation to the nucleus area shows more nuclear Cyclin D1 upon loss of *Fascin-1* or *Bbs6*. Mann-Whitney-U test. N(*Bbs6*$^{+/+}$ *siNTC*)=157, N(*Bbs6*$^{+/+}$ *siFscn1*)=132, N(*Bbs6*$^{-/-}$ *siNTC*)=157, N(*Bbs6*$^{-/-}$ *siFscn1*)=157. **C** Quantification of the fluorescence intensity of Cyclin D1 inside the nucleus of *Bbs6*$^{-/-}$ MEFs transfected with *siNTC* or *siFscn1* under lower microscopy illumination settings for Cyclin D1

shows that nuclear Cyclin D1 is significantly reduced after combined loss of *Fascin-1* and *Bbs6* in comparison to *Bbs6*$^{-/-}$ (Mann-Whitney-U test: p=0.002). N(*Bbs6*$^{-/-}$ *siNTC*)= 157, N(*Bbs6*$^{-/-}$ *siFscn1*)= 128. **D+E** Quantification of nuclear acetylated beta-catenin after loss of *Bbs6* and *Fasicn-1*. **D** Visualisation of acetylated beta-catenin inside the nucleus of *Bbs6* MEFs transfected with *siNTC* and *siFscn1* 48 h prior to fixation. Scale bars: 10µm. **E** Quantification of the fluorescence intensity of acetylated beta-catenin inside the nucleus of *Bbs6* MEFs transfected with *siNTC* or *siFscn1* measured with Fiji in relation to the nucleus area. Acetylated beta-catenin is significantly upregulated upon loss of either *Bbs6*, *Fascin-1* or both proteins combined. Mann-Whitney-U test. N(*Bbs6*$^{+/+}$ *siNTC*)=282, N(*Bbs6*$^{+/+}$ *siFscn1*)=211, N(*Bbs6*$^{-/-}$ *siNTC*)=309, N(*Bbs6*$^{-/-}$ *siFscn1*)=327. n.s. p>0.05, *p<0.05, **p<0.01, ***p<0.001. Data represent at least three independent experiments.

Discussion

In the current work, we uncover a new role for the actin-bundling protein Fascin-1 in modulating ciliary signalling. We identified that Fascin-1 forms a complex with the ciliary protein Bbs6 which might underlie the filopodia phenotype in ciliary mutants. Conversely, knockdown of Fascin-1 led to a ciliary phenotype correlating with downstream ciliary Wnt signalling.

Previous studies have shown that several ciliary proteins are not only associated with ciliary function, but also with actin regulation and subsequent cell migration (Yin et al., 2009; Kim et al., 2010; May-Simera et al., 2016). In particular loss of *Bbs6* enhances actin stress fibres and focal adhesions in kidney medullary cells, both key elements of the actin network (Hernandez-Hernandez et al., 2013). Filopodia represent another key element of the actin cytoskeleton as they extend beyond the leading edge of lamellipodia to sense the environment and are thus important to induce cell migration. Live cell imaging of MEFs revealed that filopodia in *Bbs6* knockout MEFs were significantly shorter in comparison to wildtype cells, suggesting a defect in environmental sensing. Previously, the actin phenotype upon loss of *Bbs6* or *Bbs8* was attributed to the regulation of Rho GTPases, affecting downstream actin (Hernandez-Hernandez et al., 2013). Additionally, we identified BBS6 in a complex with the actin regulator Fascin-1. As a functional downstream target of Rho signalling (Jayo et al., 2012), Fascin-1 bundles parallel actin filaments, stabilising key migratory structures such as filopodia (Kureishy et al., 2002; Vignjevic et al., 2006; Pfisterer et al., 2020). BBS8 was not found in a complex with Fascin-1, and although Bbs8 was previously associated with focal adhesions and stress fibres similarly to Bbs6 (Hernandez-Hernandez et al., 2013), we did not see a defect in filopodia length upon loss of *Bbs8*.

Our data further indicate a positive role for Fascin-1 in ciliogenesis. Although primary cilia are predominantly microtubule-based structures, actin related proteins have long been found to affect ciliogenesis. During the initial stages of ciliogenesis, many actin regulators, such as Arp2/3, focal adhesion kinase (FAK), vinculin, paxillin and Rho GTPases, are involved in the maturation of the mother centriole and positioning of the basal body (Brücker et al., 2020). In cycling cells, polymerised F-actin is associated with decreased ciliogenesis (Bershteyn et al., 2010; Kim et al., 2010, 2015; Liang et al., 2016). We were able to show that Fascin-1 joins the long list of actin-binding

proteins regulating ciliogenesis. Loss of *Fascin-1* reduced cilia numbers without affecting cilia length, suggesting a role for Fascin-1 in the initiation of ciliogenesis. Despite interaction between Fascin-1 and BBS6, we found that Fascin-1 acted antagonistically to Bbs6 on ciliogenesis, since the combined loss of both proteins rescued cilia numbers.

BBS6 is a bona fide ciliary protein. It is part of a chaperonin-like complex essential for the initial assembly of the BBSome (Seo et al., 2010), a multiprotein complex required for ciliary trafficking (Nachury et al., 2007; Wei et al., 2012). Thus, depletion of *Bbs6* correlates with a cell-type specific ciliation phenotype, reducing cilia numbers and length in RPE cells and tissue and enhancing both ciliation and ciliary length in kidney medullary cells (Patnaik et al., 2019; Volz et al., 2021). We have shown that loss of *Bbs6* in MEFs enhanced cilia numbers, although the length was not affected. Since loss of Fascin-1 also resulted in a ciliary phenotype, it is possible that Fascin-1 is required for BBS6 function in chaperoning other important ciliary trafficking proteins.

BBS6 also interacts with MACF1, an actin- and microtubule-binding protein involved in the docking of preciliary vesicles in the initial steps of ciliogenesis (May-Simera et al., 2016). Thus, the interaction between BBS6 and Fascin-1, similar to MACF1, might be also important for docking of preciliary vesicles during early ciliogenesis, a process that requires both a stable actin and microtubule network.

It is further possible that the regulation of Fascin-1 in ciliogenesis might be facilitated via its interaction with Nesprin-2, an actin-binding protein at the outer nuclear lamina that was found to be important for precise trafficking of Arp2-dependent preciliary vesicles during centriole maturation (Jayo et al., 2016; Fan et al., 2020).

Since actin proteins are highly involved in many steps of ciliogenesis, it is not surprising that F-actin itself and many actin regulators have recently been identified inside primary cilia (Nager et al., 2017; Phua et al., 2017; Kiesel et al., 2020). We were able to show that overexpressed Fascin-1 also localises to primary cilia albeit transiently, a finding supported by the studies of Phua and colleagues (Phua et al., 2017). Since one of the functions of F-actin inside cilia is thought to involve ectocytosis at the ciliary tip as a way of ciliary disassembly, it is plausible that Fascin-1 facilitates this process (Phua et al., 2017; Kiesel et al., 2020). However, we saw Fascin-1 localising along the complete axoneme and not accumulated at the ciliary tip. Besides regulating F-actin bundles, Fascin-1 is also able to bind to and regulate microtubules to control focal adhesion dynamics and speed of cell migration independently of its actin-binding function (Villari et al., 2015). This raises the possibility that the localisation of Fascin-1 inside primary cilia might be co-dependent on regulating both F-actin and microtubules, although we did not find an increase in ciliary Fascin-1 when introducing cytoskeletal mutants. Taken together, we identified Fascin-1 as a possible ciliary protein, localising transiently to primary cilia and contributing to cilia assembly. These data suggest that mutations in Fascin-1 might be associated with ciliopathies, an insinuation that is supported by findings of its second isoform, Fascin-2.

The second isoform of Fascin, retinal Fascin-2, is highly homologous to Fascin-1 and has many characteristics that are reminiscent of other ciliopathy proteins. Fascin-2 localises to the inner and outer segment of photoreceptor cells (the outer segment being a highly specialised primary cilium) and actin-based stereocilia of the cochlea (Yokokura et al., 2005; Lin-Jones and Burnside, 2007; Perrin et al., 2013). Mutations in its gene *fascin-2* are associated with retinopathies and progressive hearing loss due to shortened stereocilia bundles in mice (Yokokura et al., 2005; Perrin et al., 2013; Liu et al., 2018). There is also evidence that patient mutations in *Fascin-2* lead to macular degeneration and cone dystrophy, both common ciliopathy phenotypes (Wada et al., 2003; Gui et al., 2018). These data raise the possibility that Fascin-2, and possibly its isoform Fascin-1, the focus of this paper, are bona fide ciliopathy proteins. Since there is still a certain percentage of ciliopathy patients with undiagnosed mutations, Fascin-1 and Fascin-2 might be interesting candidates to screen in ciliopathy patients.

Since we found loss of *Fascin-1* resulting in reduced cilia assembly, we need to consider the downstream function of Fascin-1 in relation to ciliary dysfunction and actin dynamics. The signalling planar cell polarity (PCP) pathway, also referred to as the non-canonical Wnt signalling pathway, bridges ciliogenesis and actin networks. Upon activation of the PCP pathway, Frizzled receptor activation recruits Dishevelled to the plasma membrane via Inversin, where it activates formins such as Daam1 and Rho GTPases that consequently regulate downstream actin networks. BBS6 interacts with Inversin and facilitates its transport to the base of the cilium, activating non-canonical Wnt signalling (Patnaik et al., 2019). Consequently, Bbs6 is a positive regulator of non-canonical Wnt signalling (Gerdes et al., 2007; May-Simera et al., 2018; Patnaik et al., 2019; Volz et al., 2021). Concurrently, we showed that Bbs6 suppressed a downstream canonical Wnt target, Cyclin D1. Fascin-1 is also a negative regulator of Cyclin D1 transcription and nuclear localisation. We also showed that it interacts with the PCP effector protein Inversin, which is why we suppose that Fascin-1 acts in a complex with both BBS6 and Inversin in regulating Wnt signalling.

While discussing the role of Fascin-1 in Wnt signalling, its regulation in cancer cell lines has to be considered, where canonical Wnt signalling is usually enhanced (Shang et al., 2017). In several cancer types, Fascin-1 seems to be a positive regulator of Wnt signalling, which is in contrast to our data. Knockdown of *Fascin-1* in breast cancer cells reduced the expression of beta-catenin and Cyclin D1 via interaction with focal adhesion kinase (FAK), which consequently affects tumour cell growth (Barnawi et al., 2020). In the same cells, Fascin-1 was shown to interact with the non-canonical Wnt downstream target Daam1, promoting cancer cell migration (Hao et al., 2021). In human colorectal cancer, five putative TCF-binding sites in the untranslated region of the fascin-1 promoter have been identified, and the *Fascin-1* gene is transactivated via TCF/LEF transcription factors that drive canonical Wnt signalling (Vignjevic et al., 2007). However, it has to be noted that the expression of Fascin-1 is naturally enhanced in cancer cell lines (Jayo and Parsons, 2010). Complicating things further, loss of Fascin-1 in melanoblasts was associated with less Cyclin D1 positive nuclei (Ma et al., 2013). Since we have shown that Fascin-1 is a negative regulator of

canonical Wnt signalling in MEFs, we conclude that the role of Fascin-1 in Wnt signalling is highly cell-type specific.

There is also evidence for Fascin-1 in the regulation of Wnt signalling via cytonemes, specialised signalling filopodia, that act as signalling hubs for the Wnt pathway in zebrafish and non-cancerous cell lines (Routledge and Scholpp, 2019). Downstream non-canonical PCP signalling controls the emergence of cytonemes which can then transport Wnt molecules to recipient cells, inducing canonical Wnt cascades (Stanganello et al., 2015; Mattes et al., 2018; Rosenbauer et al., 2020). Moreover, cytonemes can also distinguish between different types of signals and selectively grow in the direction of a preferred Wnt signal (Junyent et al., 2020). Hence, cytonemes are important for distribution and receiving of Wnt signals. Because Fascin-1 is required for cytoneme formation (Mattes and Scholpp, 2018; Junyent et al., 2020), these data depict an involvement of Fascin-1 in Wnt signalling of non-cancerous cells via regulation of cytonemes. Since we have shown a phenotype in filopodia length upon loss of *Bbs6*, it is feasible that Bbs6 is also involved in the sensing function of cytonemes and the concomitant distribution of Wnt signals from or to other cells together with Fascin-1.

In the current work, we identified an association for Fascin-1 between ciliary proteins and actin-based filopodia sensing. We identified Fascin-1 as a bona fide ciliary protein since its loss led to a ciliary phenotype. We suggested that Fascin-1 is potentially involved in non-canonical Wnt/PCP signalling via its interaction with BBS6 and Inversin. Since PCP signalling affects filopodia formation, our data correlate ciliogenesis and associated PCP signalling with the observed filopodia phenotype in cilia mutant cells via Fascin-1. However, the mechanisms underlying ciliary function of Fascin-1, do not necessarily need to be the same as in filopodia. More research will help to understand the complex interplay between ciliogenesis, Wnt signalling and actin regulations, shedding light on how ciliopathies affect cellular homeostasis.

Material and methods

Cell culture

Primary mutant and wildtype mouse embryonic fibroblasts (MEFs) were isolated at day E13.5 from *Bbs6* null mice (Ross et al., 2005; Hernandez-Hernandez et al., 2013). Head and red organs were removed and remaining tissues trypsinized and dissociated first five times with a syringe followed by another five times dissociation with a 25µm needle. Cells were transferred into flasks and incubated in DMEM/F-12 (Thermofisher 31331093) supplemented with 1% P/ST (Thermofisher 10378016) and 10% FBS (Thermofisher 10270106). After reaching confluency, cells were immortalized according to the 3T3 immortalisation protocol (Reznikoff et al., 1973) and further cultured with constant passaging numbers. *Bbs6* cells were regularly genotyped as previously described (Ross et al., 2005). 3T3 immortalised *Bbs8* MEFs were a kind gift of the lab of Dagmar Wachten (Institute of Innate Immunity, Bonn) and isolated and

cultivated in the same way as *Bbs6* MEFs. For serum starvation (SS) experiments, cells were cultured for 24-48 h in OptiMEM (Thermofisher 11058021) prior harvest.
For pulldown assays, HEK293T cells were obtained from ATCC and cultured in DMEM-Glutamax (Thermofisher 31966047) supplemented with 10% FBS and 1% P/ST.
All cell lines were tested regularly for mycoplasma contamination.

Transfections

Plasmid transfection in HEK293T cells was conducted via Genetrap transfection reagent (made at NEI, NIH, Bethesda, MD, USA) as previously described (Patnaik et al., 2019). MEFs were transfected with Lipofectamine 2000 (Thermofisher; 11668030) according to the manufacturer's instructions and fixed or imaged after 48 h. In case of serum starvation experiments, cells were first transfected for 24 h and serum starved for another 24 h prior to fixation and experiments. Knockdowns were performed via RNA interference with Lipofectamine RNAiMax transfection reagent (Thermo Fisher; 13778150) according to the manufacturer's instructions. Mouse siRNAs for Fascin-1 (*siFscn1*) as well as non-targeting controls (*siNTC*) were obtained from IDT as Trifecta Kits (mm.Ri.Fscn1) and validated by RT-qPCR. Working concentration of each siRNA was 10nM. For *siFscn1*, siRNAs 3'-GAGACUUCUGGGUACUAUCAUUCGAAA-5' and 3'-GACGAUGAAACUGUAGCUCACCACACU-5' were used in combination. Validation of the knockdown is shown in Supp. Fig. 2.

RNA isolation and RT-qPCR

Total RNA was isolated from cells using TRIzol reagent (Thermo Fisher, 15596026) according to the manufacturer's instructions. RNA concentration and purity were measured using the NanoDrop™ 2000c Spectrophotometer (Thermo Fisher). 1000 ng of total RNA was reverse transcribed into cDNA by GoScript Probe 2-step RT-qPCR system (Promega, A5000). Quantity of respective cDNAs was determined with a StepOnePlus™ Real-Time PCR System (Applied Biosystems, 4376600) using SYBR Green (Thermo Fisher; Platinum™ SYBR™ Green qPCR SuperMix-UDG, 11733046) according to the manufacturer's recommendation. Cycling conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. Specificity of the amplified product was determined by melt curve analysis. Relative target gene expression (fold change) was normalized to *Gapdh* or *Ywhaz* (only in Bbs6 samples) and analysed by $2^{-\Delta\Delta CT}$ method.

Live cell imaging

MEFs were seeded at low confluency in imaging chambers (Ibidi, 80826) and transfected with RFP-Lifeact and EGFP-FSCN1 24 h post seeding. 48 h after transfection, *Bbs6* cells were imaged with a Leica SP8 confocal microscope with photomultipliers and a HyD detector (Leica, Bensheim, Germany) at 63x

magnification/1.40 oil (HC plan apochromat) under the conditions of 37°C supplied with 5% CO_2. Videos of *Bbs8* MEFs kept in an environmental chamber maintained at 37°C/5% CO_2 were acquired on a Nikon A1R inverted laser scanning confocal microscope (Nikon Instruments UK) combined with a 60X Plan Fluore oil immersion objective (NA 1). Excitation wavelengths of 488 nm and 561 nm (diode lasers) were used. To follow their movement, cells coexpressing both RFP-Lifeact and EGFP-Fascin-1 were imaged every 5 sec for 30 timepoints. Videos were acquired either with the Leica LAS X (version 3.5.7.23225) or Nikon NIS-Elements (v4) imaging software. Videos were processed with Fiji/ImageJ software (NIH, Bethesda, USA) as tiff stacks and the length of each filopodium per cell was measurement with the FiloQuant plugin for Fiji as previously described (Jacquemet et al., 2019).

Immunocytochemistry

Cells were seeded on glass coverslips 24 h prior transfections or treatments. 48 h after knockdown or serum starvation experiments, cells were washed with sterile PBS. According to the antibody requirements, methanol fixation (100% ice cold methanol 10 min on ice) or paraformaldehyde (4% PFA 10 min at RT) fixation was used. Following PFA fixation, quenching was performed with 50mM NH_4Cl for 10 min. Cells were permeabilized with PBSTx (PBS+0.3% TritonX) for 15 min and blocked with Fishblock blocking buffer (0.1% Ovalbumin, 0.5% Fish gelatine in PBS, 0.3% TritonX) for 1 h at RT. Primary antibodies were incubated in Fishblock over night at 4°C. Samples were washed three times 10 min with PBSTx and incubated with corresponding secondary antibodies for 1 h at RT. Samples were washed again twice with PBSTx and once with PBS before mounting coverslips on glass slides. Images were taken at RT either on a Leica SP8 confocal microscope with photomultipliers and HyD detector (HC plan apochromat 63x/1.4 oil CS2) or a Leica DM6000 microscope with a k5 sCMOS camera at 100x magnification/1.40 oil (Leica, Bensheim, Germany). Images showing filopodia were taken on a Zeiss LSM 900 with Airyscan 2 (63x/1.4 oil M27) (Carl Zeiss Microscopy, Jena, Germany). Processing, cilia length and fluorescence intensity measurements were all performed with Fiji/ImageJ software (NIH, Bethesda, USA).

Antibodies

For immunofluorescence, primary antibodies were used as follows: anti-FSCN1 (mm, 1:50; Invitrogen MA5-11483), anti-Arl13b (Rb, 1:800; Proteintech 17711-1-AP; mm, 1:200; Abcam N295B/66), anti-Actin (Rb, 1:200; Sigma A2066), anti-EGFP Living colors (Rb, 1:200; Takara Bio), anti-PCNT (1:500; Abcam Ab4448), anti-β-catenin (Rb, 1:200; Cell Signaling D10A8), anti-acetylated-β-catenin Lys49 (Rb, 1:150; Cell Signaling D7C2), anti-Cyclin D1 (Rb, 1:150; Cell Signaling E3P5S).

Secondary antibodies used for immunofluorescence were DAPI (1:8000, Carl Roth 6843), Phalloidin TRITC (1:400, Sigma P1951), Phalloidin 647 (1:40, Cell Signal 8940),

anti-mouse 488 (1:400, Invitrogen A21202), anti-mouse 555 (1:400, Invitrogen A31570), anti-rabbit 488 (1:400, Invitrogen A11034), anti-rabbit 555 (1:400, Invitrogen A21429).

For western blotting, the following antibodies were used: anti-Myc (mm, 1:1000; BD Biosciences 611013), anti-GFP (Rb, 1:1000; Chromotek Pabg1-10), anti-RFP (Rb, 1:7000; Thermofisher R10367), anti-FSCN1 (mm, 1:1000; Invitrogen MA5-11483), anti-Gapdh (mm, 1:2000; Cell Signaling 97166), anti-Actin (Rb, 1:1000; Sigma A2066), anti-Cyclin D1 (Rb, 1:1000; Cell Signaling E3P5S), anti-GSK3beta (Rb, 1:1000; Abcam Ab15580), anti-β-catenin (Rb, 1:1000; Cell Signaling D10A8), anti-acetylated-β-catenin Lys49 (Rb, 1:1000; Cell Signaling D7C2), anti-Arl13b (Rb, 1:1000; Proteintech 17711-1-AP), anti-Gt335 (mm, 1:1000; Adipogen AG-20B-0020-C100), anti-Inversin (Rb, 1:1000; Proteintech 10585-1-AP), anti-Ubiquitin (Rb, 1:500; Sigma-Aldrich 07-375)

Secondary antibodies for western blotting were anti-rabbit 680nm (1:10,000, LI-COR Biosciences 925-68073), anti-rabbit 800nm (1:10,000, LI-COR Biosciences 926-32211), anti-mouse 680nm (1:10,000, LI-COR Biosciences 925-68072), anti-mouse 800nm (1:10,000, LI-COR Biosciences 925-32212).

Primers RT-qPCR

Gene	**Forward**	**Reverse**
mGapdh	AATGGTGAAGGTCGGTGTGAA	AGGTCAATGAAGGGGTCGTTG
mYwhaz	TCTTGATCCCCAATGCTTCG	AATGCTTCTTGGTATGCTTGC
mBbs6	GTGTGCTCTGCAAGATTTGG	AAGACGTGCATTGCTGTTTG
mInversin	TCGCTGATGGAAACCTAACG	AAGGAGATGGACAATCTGTGC
mArl13b	CTGGGATGTTCAGTCTGATGG	TCTCCTTGGATTCCCTTTGC
mFascin-1	GTTGGAATTCAATGACGGCG	ACCTTGAGAGCCACCTTATTG
mCyclind1	TGCCATCCATGCGGAAA	AGCGGGAAGAACTCCTCTTC
mmyc	GCTGTTTGAAGGCTGGATTTC	GATGAAATAGGGCTGTACGGAG
mLrp5	GGGTCCACAAGGTCAAGGC	GCACCCTCCATTTCCATCC
mLrp6	GCCCACTACTCCCTGAATGCTG	TGTGGATAGGAAGGATGATGTCAGG
mDvl2	GGCTTGTGTCGTCAGATACC	TTTCATGGCTGCTGGATAC
mDvl3	CCGATGAGGATGATTCCACC	TGAGGCACTGCTCTGTTCTG
mAxin2	GAGTAGCGCCGTGTTAGTGACT	CCAGGAAAGTCCGGAAGAGGTATG
mβ-catenin	GTGCAATTCCTGAGCTGACA	CTTAAAGATGGCCAGCAAGC
mLef1	GTCCCTTTCTCCACCCATC	AAGTGCTCGTCGCTGTAG
mβ-actin	CACAGCTGAGAGGGAAATCGTGC	GATCTTGATCTTCATGGTGCTAGG

Pulldown assays and western blotting

For interaction studies, HEK293T cells were co-transfected with EGFP-FSCN1, mRFP-FSCN1, EGFP-INVS, pCMV-MYC-BBS8 or pCMV-MYC-BBS6 or empty vector

controls using Genetrap as described above. After 48 h, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% Sodiumdeoxycholate, 0.1% SDS) containing Halt™ Protease and Phosphatase Inhibitor Cocktail (100X, Thermo Fisher 78442). Pulldowns for EGFP were performed with magnetic agarose beads (GFP-Trap-MA, ChromoTek) according to the manufacturer's instructions. Proteins were washed off the beads with 1X Laemmli loading buffer containing SDS, DTT and beta-mercaptoethanol at 95°C for 10 min. For co-immunoprecipitations of Fascin-1 and Ubiquitin, MEFs were treated with MG132 (10µM; Calbiochem 474791) for 5 h before lysates were prepared as described above. Immunoprecipitations were carried out with 12.5 µl Dynabeads Protein-G (Fisher Scientific, 10003D) per sample, washed with 500 µl PBSTx (0.01% TritonX) and incubated for 4 h at 4°C with 6 µl Fascin antibody (mm, Invitrogen MA5-11483) or mouse IgG control during rotation. Lysates were incubated rotating on antibody coated beads overnight at 4°C. Beads were washed 3 times with PBSTx and proteins were eluted with 1X Laemmli buffer without DTT and beta-mercaptoethanol at 95°C for 10 min. Before loading on gel, DTT and beta-mercaptoethanol was added again to avoid protein aggregates.

Proteins were separated on 10% polyacrylamide gels via SDS-PAGE followed by western blotting. Proteins were transferred onto PVDF membranes (Immobilon®-FL PVDF membrane, Sigma, 05317) and blocked with Applichem blocking buffer (0.2% AppliChem Blocking Reagent, 10 mM TrisHCl, 150 mM NaCl, 0.04% NaN_3; pH 7.4) or 5% milk or 5% BSA blocking according to the antibody's requirement. Membranes were probed with antibodies over night at 4°C, washed with TBS 0.1% Tween and incubated with corresponding secondary antibodies for 1 h at RT. Scanning was performed in the Odyssey Infrared Imaging System (Licor) at 680 or 800 nm. Densitometry analysis (AUC) was performed with Fiji/ImageJ software (NIH, Bethesda, MD, USA) and the expression levels were normalised to the inputs or Gapdh expression level.

Statistical analysis

Statistical analysis was performed using IBM SPSS 27 software (IBM, NY, USA). Parametric or non-parametric data distribution was determined using Shapiro-Wilk test and outliers were extracted. Parametric differences were determined using t-test. Differences between two non-parametric groups were compared using a Mann-Whitney-U/Wilcoxon signed-rank test. P-values of 0.05 and below were considered statistically significant. Statistical tests and number of repetitions are described in the legends. Boxplots show median (middle line), edge of boxes is top and bottom quartiles (25–75%), and whiskers represent the ranges for the upper 25% and the bottom 25% of data values. Outliers are shown as circles above and below whiskers. Bar plots show mean ± standard errors (SE).

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Declaration of interest

The authors have no competing interest to declare.

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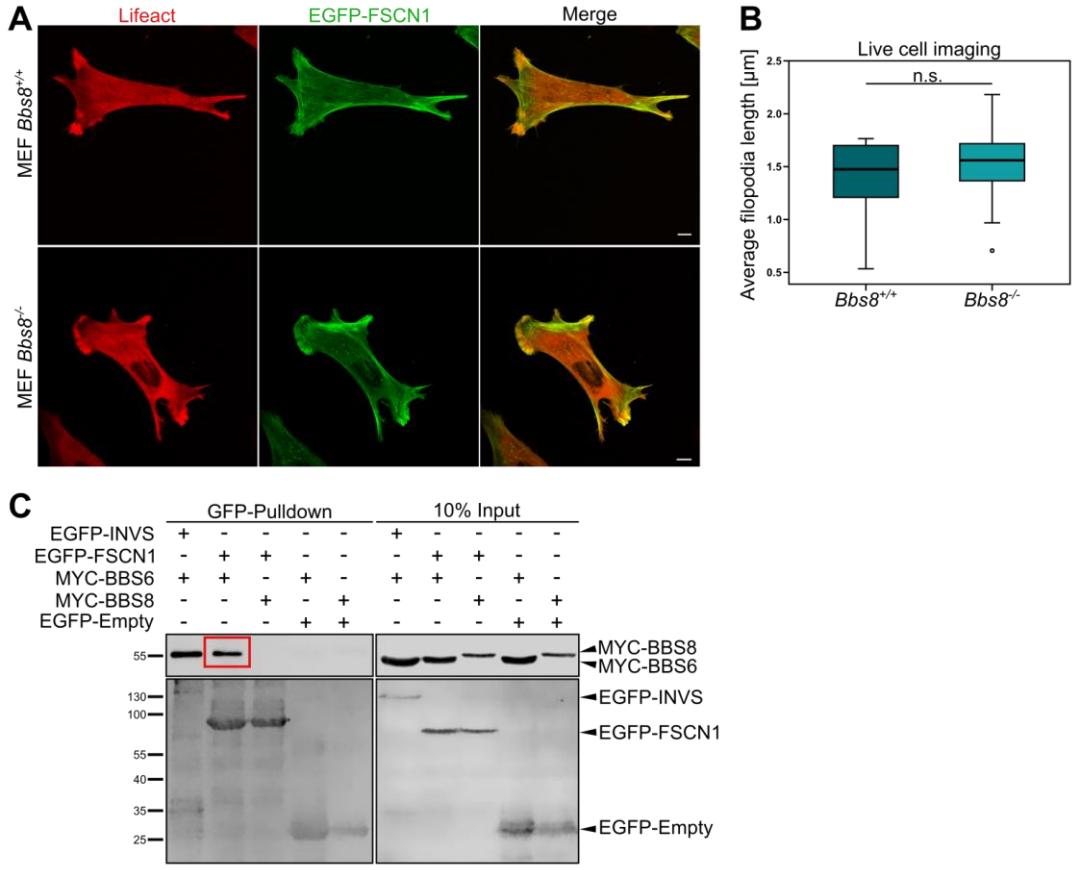
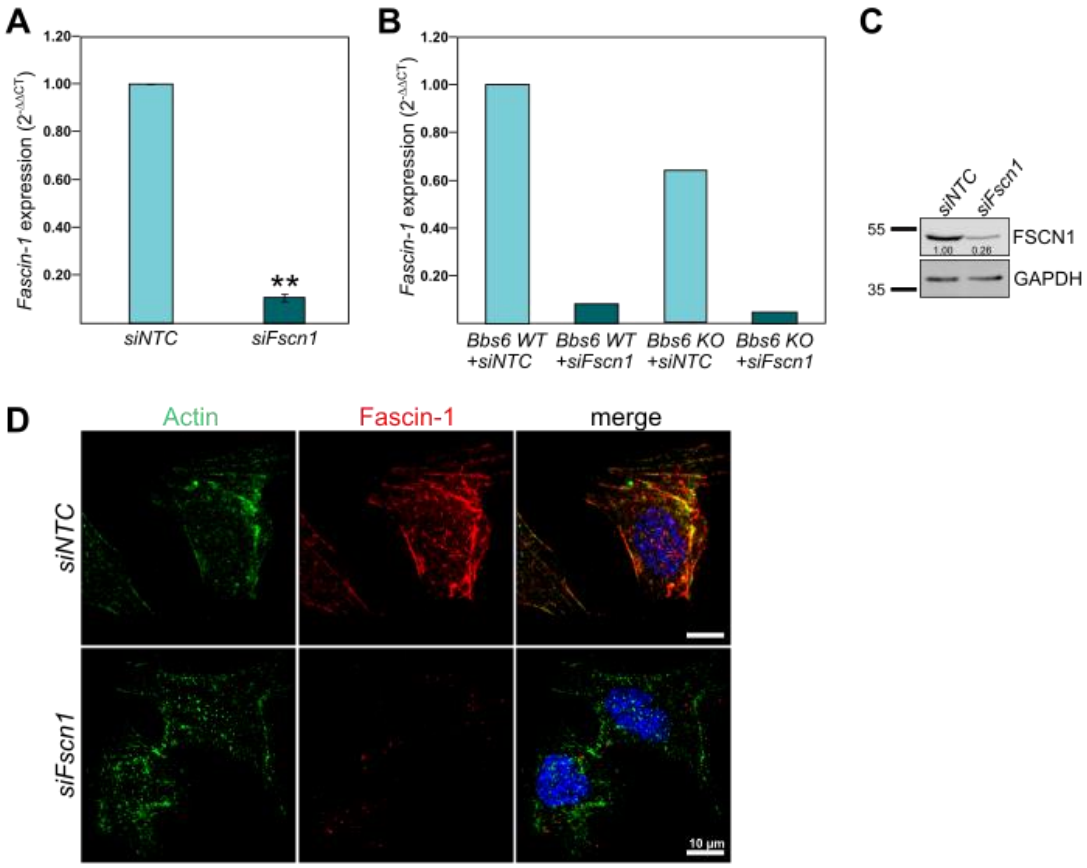
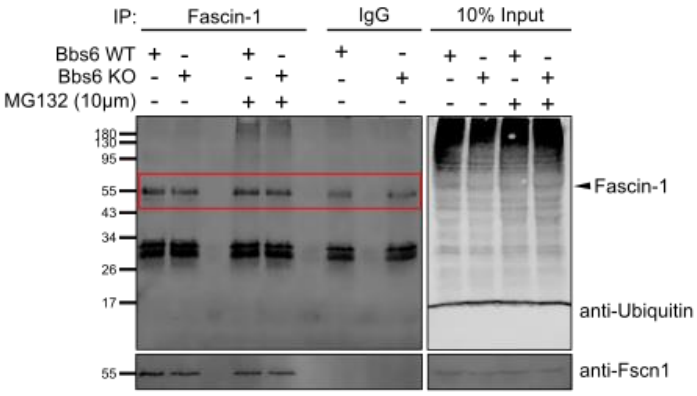
Supplementary figures



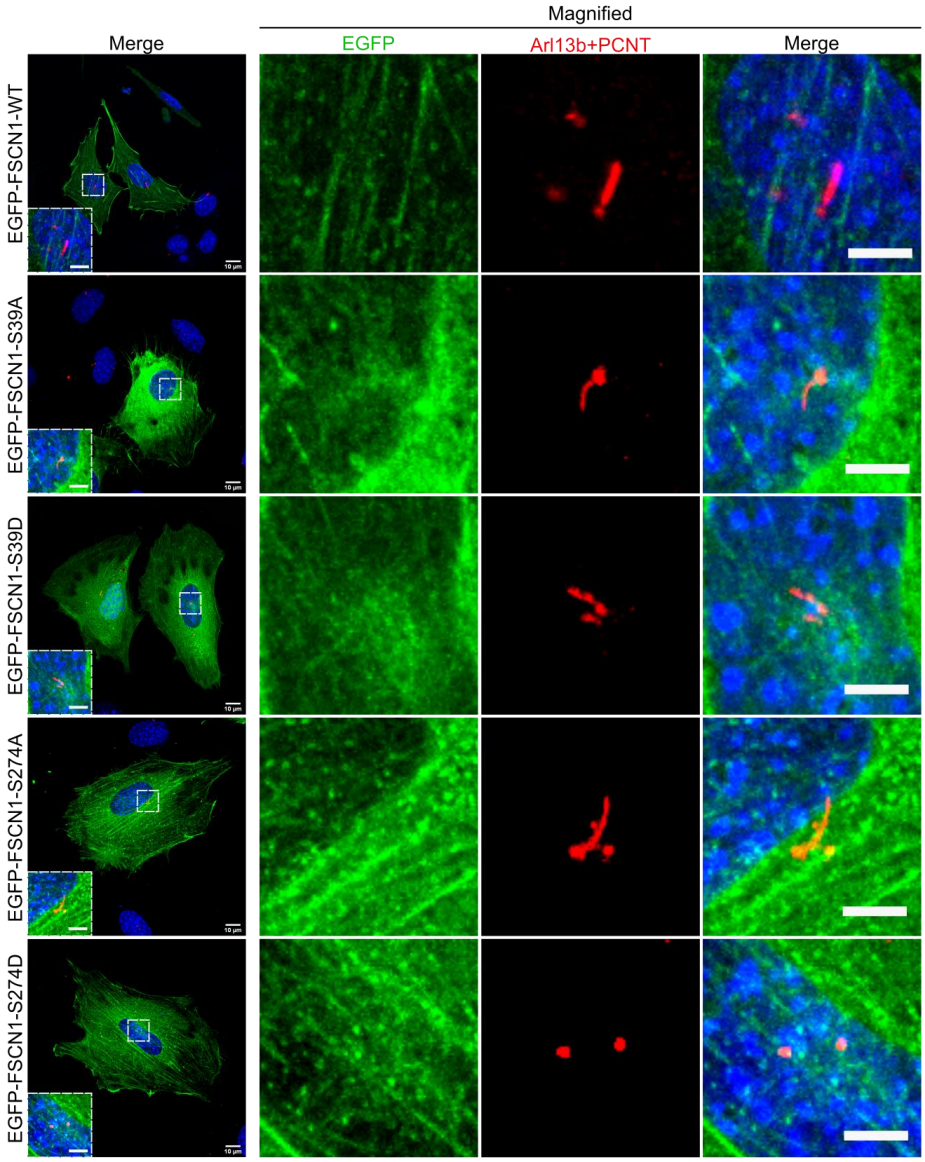
Supp. Fig. 1: Bbs8 does not affect filopodia dynamics.
A Live cell imaging of *Bbs8* MEFs overexpressing Lifeact for the actin cytoskeleton and EGFP-FSCN1 as a filopodia marker. Images represent one timepoint out of 30, videos are shown in supplementary videos 3 and 4. **B** Average filopodia length via FiloQuant analysis shows no significant difference in filopodia length upon *Bbs8* knockout (Mann-Whitney-U test p=0.74). N(*Bbs8*$^{+/+}$)=18, N(*Bbs8*$^{-/-}$)=16. Experiments were repeated twice. **C** Interaction study between Fascin-1 and BBS6/BBS8. GFP pulldowns were performed 48 h after overexpression of EGFP-FSCN1 and MYC-BBS6 or MYC-BBS8 in HEK293T cells. The interaction between EGFP-INVS (Inversin) and MYC-BBS6 was used as positive control, EGFP-empty served as negative control. MYC-BBS8 did not form a complex with EGFP-FSCN1. Red box shows positive interaction between EGFP-FSCN1 and MYC-BBS6 as shown in figure 1.



Supp. Fig. 2: Validation of Fascin-1 knockdown in mouse embryonic fibroblasts.
A mRNA level of *Fascin-1* ($2^{-\Delta\Delta CT}$) in MEFs 48 h after knockdown measured by RT-qPCR. mRNA expression of *Fascin-1* is significantly reduced to ~11% after knockdown in comparison to *siNTC*. N=3. **B** mRNA level of *Fascin-1* ($2^{-\Delta\Delta CT}$) in *Bbs6* wildtype and knockout MEFs 48 h after knockdown measured by RT-qPCR shows a successful reduction in mRNA of *Fascin-1* in both cell lines. N=3. **C** Protein expression of Fascin-1 in MEFs validated by western blot is reduced to 26% after knockdown of *Fascin-1*. Numbers show AUC for protein bands in relation to Gapdh and normalised to *siNTC* (fold change). **D** Visualisation of Fascin-1 (red) via endogenous immunofluorescence staining in MEFs reveals less signal for Fascin-1 after *Fascin-1* knockdown.



Supp. Fig. 3: Bbs6 does not affect ubiquitination of Fascin-1.
Immunoprecipitation of endogenous Fascin-1 of *Bbs6*$^{+/+}$ and *Bbs6*$^{-/-}$ MEFs with or without treatment of MG132 (5 h). Probing the membranes with Ubiquitin antibody reveals no differences between level of ubiquitinated Fascin-1 (red box) in Bbs6 wildtype or knockout cells. N=2.



Supp. Fig. 4: Localisation of EGFP-tagged Fascin-1 mutations in MEFs.
Overexpression of EGFP-tagged FSCN1 variants (green) and costaining with cilia markers PCNT (basal body) and Arl13b (cilia membrane; both red) 48 h after transfection in serum-depleted MEFs shows no improvement of ciliary localisation of the different mutations. Scale bars of magnified images: 3µm.

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Review article

The entangled relationship between cilia and actin

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Ciliopathies

ABSTRACT

Primary cilia are microtubule-based sensory cell organelles that are vital for tissue and organ development. They act as an antenna, receiving and transducing signals, enabling communication between cells. Defects in ciliogenesis result in severe genetic disorders collectively termed ciliopathies. In recent years, the importance of the direct and indirect involvement of actin regulators in ciliogenesis came into focus as it was shown that F-actin polymerisation impacts ciliation. The ciliary basal body was further identified as both a microtubule and actin organising centre. In the current review, we summarize recent studies on F-actin in and around primary cilia, focusing on different actin regulators and their effect on ciliogenesis, from the initial steps of basal body positioning and regulation of ciliary assembly and disassembly. Since primary cilia are also involved in several intracellular signalling pathways such as planar cell polarity (PCP), subsequently affecting actin rearrangements, the multiple effectors of this pathway are highlighted in more detail with a focus on the feedback loops connecting actin networks and cilia proteins. Finally, we elucidate the role of actin regulators in the development of ciliopathy symptoms and cancer.

1. Introduction

Primary cilia are microtubule-based cellular membrane protrusions that are important for signalling and communication within numerous cells and tissue types. Over the last two decades, it has been shown that primary cilia defects underlie several genetic disorders, collectively referred to as ciliopathies. Until now, more than 20 syndromic ciliopathies such as Bardet-Biedl syndrome (BBS), Meckel-Gruber syndrome (MKS), Joubert syndrome (JS), McKusick-Kaufman syndrome (MKKS) or Nephronophthisis (NPHP) have been described, sharing multiple overlapping phenotypes such as retinopathies, kidney cysts, obesity or polydactyly (Forsythe et al., 2018; Fraser and Davey, 2019; Hartill et al., 2017; Praveen et al., 2015; Slavotinek and Biesecker, 2000; Srivastava et al., 2018). Non-syndromic ciliopathies in which only one organ or tissue type is affected, for example polycystic kidney disease (PKD) or retinitis pigmentosa (RP), are more common (Bergmann, 2015; Estrada-Cuzcano et al., 2012).

The overall structure of a primary cilium consists of the basal body, derived from the mother centriole, which anchors the cilium inside the cell, and the ciliary axoneme (Fig. 1). The basal body, embedded in a cloud of pericentriolar material (PCM), is composed of nine microtubule triplets connected to the intracellular microtubule cytoskeleton via subdistal appendages (Delgehyr et al., 2005; Tateishi et al., 2013). In ciliated cells, the basal body represents the microtubule organising centre (MTOC) of the cell as it nucleates and anchors microtubules (Delgehyr et al., 2005). Nine microtubule doublets extend from the basal body into the ciliary axoneme. The cilium is built, maintained and subsequently disassembled via intraflagellar transport (IFT), a cargo machinery that transports proteins into and out of the cilium (Ishikawa and Marshall, 2017; Kozminski et al., 1993).

Historically, primary or sensory cilia, occurring solitary per cell, were thought to be strictly separable from motile cilia, of which there can be multiple per cell, and which are required for fluid transport over membrane surfaces (Lee, 2011). This initial distinction stems from structural differences between primary and motile cilia. However, recent data indicate that motile cilia also execute sensory functions (Bloodgood, 2010; Jain et al., 2012; Shah et al., 2009).

Although primary cilia are predominantly microtubule-associated structures, recent studies implicate a prominent role for F-actin and actin-associated proteins in cilia processes. Filamentous actin (F-actin) is composed of polarised globular G-actin monomers that aggregate and twist around each other to form stable F-actin filaments (Dominguez and Holmes, 2011). These F-actin filaments were found to play important roles in many cellular processes as their rearrangement is tightly connected to cell cycle and cell migration. The current review will focus on recent research investigating the involvement of F-actin during

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ciliogenesis, disassembly and its contribution to ciliopathy phenotypes and cancer.

2. Actin during ciliogenesis

Ciliogenesis is a highly complex process that can either occur via an extracellular pathway in which initiation begins at the cell surface or an intracellular pathway starting within the cytoplasm (Wang and Dynlacht, 2018). Most research has focused on the intracellular pathway, which requires several different proteins during centriole maturation, basal body docking and subsequent axonemal growth, with F-actin being involved in many of these processes.

As cells exit the cell cycle, the mother centriole undergoes maturation to initiate cilia formation. During this process, small preciliary vesicles, originating from the Golgi and endosome, are transported along microtubules and dock at the distal appendages of the mother centriole (Schmidt et al., 2012; Wu et al., 2018). The transport and subsequent docking events of preciliary vesicles are particularly dependent on an intact F-actin network and the actin-dependent motor proteins myosin Va and myosin heavy chain 10 (Hong et al., 2015; Wu et al., 2018). Hereby, actin filament nucleation is initiated via the actin nucleator complex Arp2/3, which is recruited via pericentriolar material-1 (PCM-1) (Farina et al., 2016; Obino et al., 2016). This involves the interaction and recruitment of Rab GTPases such as Rab11, Rabin8 and the small GTPase Rab8, which are commonly known for actin regulation (Wu et al., 2018). Rab GTPases in general play a prominent role in centriole maturation as reviewed in Blacque et al. (2018).

After docking of preciliary vesicles at the mother centriole, distal appendage vesicles fuse to form the ciliary vesicle, a process involving membrane tubulation (Lu et al., 2015). This remodelling of the distal ends of the mother centriole results in the extension of axonemal microtubule doublets. This nascent primary cilium docks at the plasma membrane via its distal appendages where it is anchored to the intracellular microtubule network via subdistal appendages (Huang et al., 2017). In addition, focal adhesion complexes including proteins such as focal adhesion kinase (FAK), vinculin and paxillin connect the basal body to the actin cytoskeleton (Antoniades et al., 2014). During centrosome positioning and basal body docking, a RhoA-dependent apical network is formed (Pan et al., 2007). Nesprin-2, an actin-binding protein at the outer-nuclear membrane, was shown to regulate centriole migration and positioning through remodelling of RhoA-dependent actin networks via interaction with the transmembrane cilia protein MKS3 (Meckel-Gruber syndrome 3) (Dawe et al., 2009). Besides Arp2/3, the actin nucleator Cobl (Cordon-Bleu) was further shown to be critical for centrosome positioning (Haag et al., 2018). After docking at the plasma membrane, distal appendages recruit further

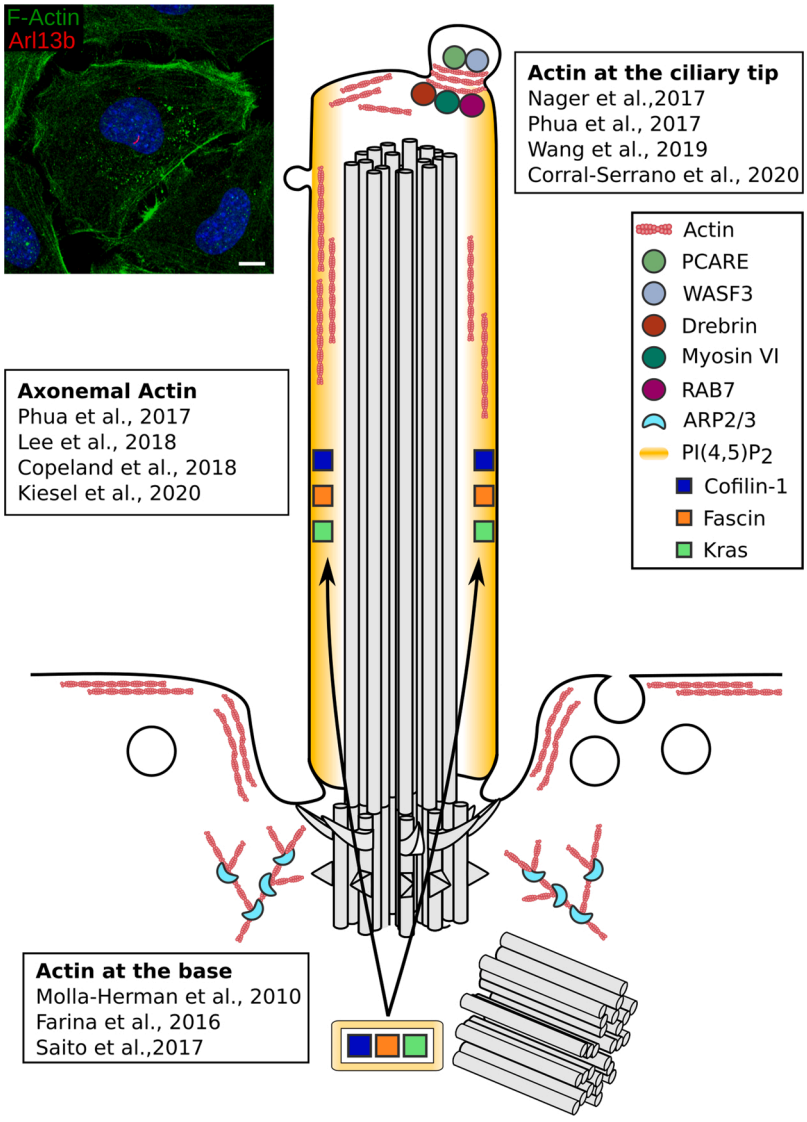

Fig. 1. Ciliary actin localisation. Top left: The immunofluorescence image shows a primary cilium visualised via Arl13b, which labels the ciliary axoneme (red), and the actin cytoskeleton (green) in mouse embryonic fibroblasts. Scale bar: 10 μm. Actin filaments (red) are found at the ciliary pocket, an evagination of the plasma membrane at the base of the cilium (Saito et al., 2017). An Arp2/3 branched actin network is also found around the basal body (Farina et al., 2016; Molla-Herman et al., 2010). F-actin was further visualised in the ciliary axoneme (Copeland et al., 2018; Kiesel et al., 2020; Lee et al., 2018; Phua et al., 2017) and at the ciliary tip, where it is involved in cilia decapitation via ectocytosis (Corral-serrano et al., 2020; Nager et al., 2017; Phua et al., 2017; Wang, Hu et al., 2019). Actin and the actin regulator WASF3 get recruited via PCARE into the cilium to enable ectocytosis, which further involves actin regulators Drebrin, Myosin VI and Rab7. The phospholipid PI(4,5)P_2 recruits Cofilin-1, Fascin and Kras inside the cilium to support actin-based ciliary ectocytosis. PCARE: Photoreceptor Cilium Actin Regulator, WASF3: Wiskott-Aldrich Syndrome Protein Family Member 3, FHDC1: FH2 Domain Containing 1/Inverted Formin-1, Arp2/3: Actin Related Protein2/3, PI(4,5)P_2: Phosphatidylinositol 4,5-bisphosphate.

components of the intraflagellar transport (IFT) machinery to the cilium, which are, besides their role in diffusion of tubulin molecules, required for subsequent elongation of axonemal microtubules (Harris et al., 2020; Yang et al., 2018). A complex of several proteins named the BBSome, mutations in which cause the ciliopathy Bardet-Biedl syndrome, is responsible for assembling IFT complexes with their cargos in order to build and maintain cilia by trafficking of tubulin and signalling molecules (Blacque et al., 2004; Wei et al., 2012). Following basal body docking, the ciliary pocket is formed via membrane remodelling which is also driven by actin proteins (Saito et al., 2017).

The initiation of ciliogenesis and centrosome positioning requires both a stable actin and microtubule network (Pitaval et al., 2017, 2010). Therefore, proteins regulating both networks have recently been investigated in relations to ciliogenesis. The inverted formin-1 (FHDC1) is one of the only mammalian formins affecting early ciliogenesis via interaction with subdistal appendage protein Cep170 (Copeland et al., 2018). Unlike other formins, known to nucleate actin, FHDC1 also functions in microtubule stabilisation and acetylation via its FH1-FH2 domain (Thurston et al., 2012; Young et al., 2008). Due to this combined regulation of actin and microtubules and its interaction with Cep170, depletion of FHDC1 resulted in inhibition of ciliogenesis, whereas overexpression lead to defects in ciliary disassembly and accumulation of F-actin inside cilia (Copeland et al., 2018). Another protein regulating both actin and microtubule dynamics is the microtubule actin crosslinking factor 1 (MACF1), which has been shown to be involved in the early stages of ciliogenesis. MACF1 facilitates microtubule organisation at focal adhesions and stabilises F-actin (Antonellis et al., 2014; Wu et al., 2008). Deletion of MACF1 leads to increased actin stress fibre formation and loss of microtubule anchoring which concludes in failed basal body docking of ciliary vesicles (May-Simera et al., 2016). MACF1 was further shown to interact with the ciliopathy protein BBS6 and Talpid3, a protein that is important for recruiting the small GTPase Rab8 during preciliary vesicle docking (Kobayashi et al., 2014; May-Simera et al., 2016; Wang et al., 2016). Interestingly, loss of Talpid3 disorganises actin networks and more recently, it has been shown to regulate the assembly of subdistal appendages (Wang et al., 2018).

The acetylation of microtubules in the ciliary axoneme is mainly facilitated by the acetyltransferase Mec-17 (αTAT1), which can be reversed during ciliary disassembly by the deacetylase HDAC6 (Kim et al., 2013; Ran et al., 2015; Shida et al., 2010). Besides regulating ciliogenesis by acetylation of microtubules, further studies found that Mec-17 upregulates the expression of myosin IIB which promotes ciliogenesis in an actin dependent manner (Rao et al., 2014). Myosin IIB organises the pericentrosomal preciliary compartment (PPC) including CEP290 and PCM-1 by increasing actin dynamics. These data suggest that Mec-17 impacts the early steps of ciliogenesis via a combined approach of influencing microtubule acetylation directly and regulating actin regulators transcriptionally.

The regulation of both actin and microtubule dynamics during ciliogenesis is often attributable to regulators of PCP signalling, which will be discussed in section 5.

3. F-actin in cilia function

To what extend actin is present in the cilium is still a topic of debate. Due to technical limitations and high levels of cytoplasmic actin making visualisation in the cilium challenging, only few studies have been able to identify ciliary F-actin (summarised in Fig.1). An Arp2/3 branched F-actin network is found at the ciliary base and the ciliary pocket acts as an interface between stable and dynamic actin filaments (Farina et al., 2016; Molla-Herman et al., 2010; Saito et al., 2017). However, actin composition within the cilium is likely to be cell type and cell cycle dependent. Early on, F-actin was shown to be present in the connecting cilium of photoreceptor cells, representing the transition zone of this highly specialised primary cilium (Chaitin et al., 1984; Williams, 1991; Woodford and Blanks, 1989). In epithelial cell culture, Lee et al. were able to visualise F-actin inside cilia of cultured mammalian IMCD3 cells upon maximising microscope intensity (Lee et al., 2018). In concordance with this, a recent landmark study by Kiesel et al. was finally able to identify filamentous actin inside the axonemes of primary cilia in MDCK-II cells using both cryo-electron and confocal microscopy (Kiesel et al., 2020). In support of both studies, ciliary proteomic studies have identified several actin-associated proteins (Ishikawa et al., 2012; Kim et al., 2010; Kohli et al., 2017; Mick et al., 2015). However, only two studies have actually focused on these findings in a proteomic screen of the ciliary membrane (Kohli et al., 2017) and in a siRNA-based genomic screen (Kim et al., 2010).

More recently, studies examining a role for actin in cilia have focused on its potential function during cilia decapitation. The process of cilia decapitation or shedding via ectocytosis of extracellular vesicles, so-called ectosomes, represents an alternative actin-dependent cilia disassembly mechanism as well as an alternative method for removing ciliary components (Mirvis et al., 2019; Phua et al., 2017; Wang and Barr, 2018).

The process of ectocytosis can be reconstructed in the outer segments of photoreceptor cells which represent highly specialised primary cilia. In *rds* mutant mice, disc formation is inhibited due to a lack of the protein peripherin which means that the evaginating membrane is shed in form of ectosomes (Connell et al., 1991; Salinas et al., 2017; Travis et al., 1991). This phenotype is common in other cilia mutant photoreceptors and represents a good example for studying proteins involved in ciliary membrane evagination (Dilan et al., 2018). Proteomic investigation of these abnormally released ectosomes identified actin regulators such as C2orf71/PCARE, WASF3 and members of the Arp2/3 complex, indicating the involvement of these proteins in ciliary ectocytosis (Spencer et al., 2019).

Furthermore, actin regulators drebrin and myosin VI have been shown to localise to the ciliary tip in cultured mammalian IMCD3 cells pointing to a role of actin at the site of cilia decapitation (Nager et al., 2017, see Fig. 1). Drebrin and myosin VI were shown to mediate the ectosomal release of ciliary G-protein coupled receptors (GPCRs) at the ciliary tip upon failed 'normal' BBSome-dependent retrieval out of the cilium (Nager et al., 2017). Actin depolymerising drugs such as Cytochalasin D or Latrunculin abolished the scission of ectosomes, which underlines the importance of actin in this process (Nager et al., 2017; Phua et al., 2017).

In accordance with this, Phua et al. were able to visualise phalloidin-stained F-actin at the spot of cilia excision in murine fibroblasts (Phua et al., 2017). They suggested that the actin regulators cofilin-1, fascin and the small GTPase Kras are recruited via the phospholipid $PI(4,5)P_2$ to induce actin polymerisation at the site of primary cilia scission (Phua et al., 2017).

The finding that polymerised F-actin at the ciliary tip is essential for ectocytosis was recapitulated in more recent studies performed in hTERT-RPE-1 cells (Corral-serrano et al., 2020; Wang, Hu et al., 2019). Wang et al. found this process to be dependent on direct binding between F-actin and the small GTPase Rab7. Corral-Serrano et al. showed that F-actin localisation throughout the primary cilium can be triggered by overexpression of C2orf71/PCARE (photoreceptor cilium actin regulator), which recruits F-actin together with the Arp2/3 complex activating factor WASF3 to the cilium (Corral-serrano et al., 2020). This led to actin-positive ciliary membrane expansions at the ciliary tip which likely proceeds the process of cilia decapitation or ectocytosis. This is in line with studies by Spencer et al. indicating C2orf71/PCARE, WASF3 and Arp2/3 being part of ectosomes shed by mutant photoreceptor cells as discussed above (Spencer et al., 2019). Another study in murine NIH-3T3 fibroblasts indicated that overexpression of the formin FHDC1 traps F-actin inside elongated cilia upon defective cilia disassembly (Copeland et al., 2018); however, formin activity seems to be dispensable for ciliary decapitation (Nager et al., 2017).

Taken together, these studies indicate that F-actin might be localised in primary cilia of multiple cell types, but its dynamic localisation and

cell-type specific expression restricted its visualisation in cilia for a long time. Its function in ciliary decapitation includes several key actin regulators and there might be many more actin regulators involved in cilia decapitation processes as suggested by proteomic screens. Ciliary ectocytosis might also be possible at the basal part of the ciliary membrane or the ciliary base (Hogan et al., 2009; Huang et al., 2016; Pampliega et al., 2013), a process which might also involve actin. An evidence for this is the direct interaction between actin and the Arf-like small GTPase Arl13b (Barral et al., 2012), which is associated with the ciliopathy Joubert syndrome and is commonly used as a specific ciliary membrane marker (Cevik et al., 2010). It might be plausible that Arl13b also recruits actin inside the cilium. In some of the studies discussed above, actin is found throughout the cilium and not just at the site of ciliary abscission, thus actin might function in other processes at the transition zone or ciliary pocket.

4. Actin dynamics and the effect on ciliation

Besides the novel role of actin in ciliary decapitation, numerous studies observed an antagonistic effect of actin polymerisation on ciliogenesis. Blocking actin polymerisation via inhibitors such as cytochalasin D or latrunculin B stabilises primary cilia, preventing ciliary disassembly (Kim et al., 2010; Pitaval et al., 2010). Concordant studies showed that F-actin depolymerising proteins such as cofilin-1 or gelsolin increases ciliogenesis (Kim et al., 2010; Kim et al., 2015). Since F-actin polymerisation occurs during ciliary disassembly, it was suggested that the presence of polymerised actin suppresses ciliogenesis potentially via inducing ciliary disassembly components (Bershteyn et al., 2010; Liang et al., 2016). A graphical representation of actin regulators involved in ciliary assembly or disassembly is given in Fig. 2.

The polymerisation of F-actin is regulated by various regulators and is downstream of several signalling pathways. One example is PCP signalling which will be discussed in section 5. The often overlapping and interconnected signalling cascades involve key actin regulators such as actin nucleators or capping proteins, but also GTPases, histone modifiers, transcriptional regulators as well as micro-RNAs. Their complex crossover in activity impacts underlying actin dynamics and subsequently cilia stability in various mechanisms or feedback loops. One of the key actin regulators involved in several cilia disassembly mechanisms is the Arp2/3 complex. The Arp2/3 complex is a well-characterised actin nucleator essential for organising actin filaments into branched actin networks. Arp2/3 was shown to nucleate actin filaments at centrosomes and to facilitate ciliary vesicle transport to the basal body (Farina et al., 2016). Suppression of actin polymerisation via Arp2/3 inhibition results in immobilisation of IFT trains at the ciliary tip, promoting ciliogenesis (Cao et al., 2012; Kim et al., 2010; Yeyati et al., 2017). Combined, these functions of Arp2/3 suggest that it acts as a negative regulator of ciliogenesis.

Actin polymerisation also plays a role in HDAC6 mediated ciliary disassembly, most likely via antagonism between the branched actin regulators cortractin and missing-in-metastasis (MIM) (Bershteyn et al., 2010; Ran et al., 2015). The histone deacetylase HDAC6 mediates ciliary disassembly via deacetylation of cortractin, a Arp2/3 complex activator, and alpha-tubulin (Ran et al., 2015). MIM inhibits actin filament nucleation directly through interaction with actin and on the other hand, promotes actin polymerisation via interaction with cortractin, N-WASP and the GTPase Rac1 (Bompard et al., 2005; Lin et al., 2005; Mattila et al., 2003; Yamagishi et al., 2004). It was further shown that MIM stabilises primary cilia in a complex way via regulating the interaction between Cdc42 and the polarity protein aPKC (Drummond et al., 2018). The GTPase Cdc42 promotes N-WASP and Arp2/3 mediated actin nucleation through Toca-1 and is therefore one of the key actin regulators. It further recruits both MIM as well as the polarity complex of aPKC/Par3/Par6 to the base of the cilium where they influence Hedgehog signalling and ciliogenesis in an epistatic manner (Drummond et al., 2018). Cdc42 and Arp2/3 have further shown to be

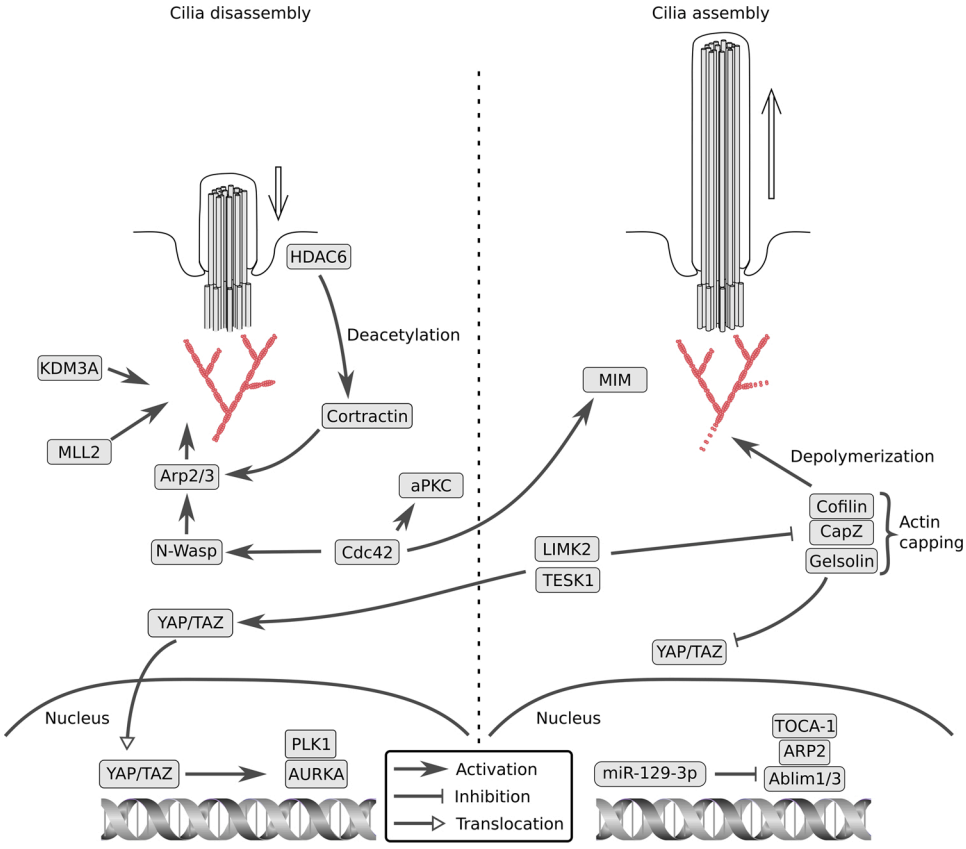

Fig. 2. Graphical representation of the crosstalk between actin regulatory proteins during ciliary assembly and disassembly. Several actin regulators lead to ciliary disassembly via polymerisation of actin filaments (red) or transcriptional activation of YAP/TAZ, which transcribe disassembly factors such as AURKA or PLK1. Upon actin depolymerisation induced by actin capping proteins, which also inhibit YAP/TAZ translocation into the nucleus, primary cilia are assembled. HDAC6: Histone deacetylase 6, KDM3A: Lysine Demethylase 3A, MLL2: Mixed-lineage Leukemia Protein 2, Arp2/3: Actin Related Protein2/3, N-Wasp: Neural Wiskott-Aldrich Syndrome Protein, Cdc42: Cell Division Cycle 42, aPKC: Atypical Protein Kinase C, YAP: Yes-associated Protein, TAZ: Transcriptional Coactivator with PDZ-binding Motif, PLK1: Polo Like Kinase 1, AURKA: Aurora Kinase A, MIM: Missing-in-Metastasis, LIMK2: Lim Domain Kinase 2, TESK1: Testis Associated Actin Remodelling Kinase 1, CapZ: Capping Actin Protein Of Muscle Z-Line, TOCA-1: Transducer Of Cdc42-Dependent Actin Assembly Protein 1, ABLIM: Actin Binding LIM Protein.

influenced by Tctex-1, a component of the cytoplasmic dynein 1 complex (Saito et al., 2017). Dynein independently, Tctex-1 was shown to accelerate clathrin-mediated endocytosis at the ciliary pocket through interaction and activation of Arp2/3 and Cdc42 and is therefore crucial during early steps of ciliary disassembly.

Besides HDAC6, recent data indicated two other histone modifiers to be involved in negative regulation of ciliogenesis. KDM3A, a multifunctional protein histone lysine demethylase, was shown to recapitulate the inhibiting effect of Arp2/3 on ciliogenesis (Yeyati et al., 2017). *Kdm3a*$^{-/-}$ mice mirror phenotypes associated with human ciliopathies and *KDM3A*$^{-/-}$ cells exhibit longer and instable cilia. KDM3A directly binds to actin and transcriptionally activates its expression (Yeyati et al., 2017). Since increased actin polymerisation reverses the KDM3A phenotype, the authors attribute the cilia phenotype on loss of actin dynamics at the ciliary base. The histone lysine methylase, mixed-lineage leukemia protein 2 (MLL2) also seems to play a negative role on ciliogenesis via affecting actin proteins. Depletion of MLL2 in RPE1 cells increased both ciliary length and number (Yang et al., 2019), and patients with mutations in *MLL2* resemble the phenotype of ciliary dysfunction-associated coronary heart disease (Ang et al., 2016; Digilio et al., 2017). It was shown that MLL2 facilitates gene expression of the actin-associated proteins formin1, the CDC42 effector protein CDC42EP3, myosin 5b, synaptopodin and cofilin2 which subsequently affect ciliogenesis via Arp2/3 mediated branched actin regulation.

The transcriptional coactivators YAP/TAZ (Yes-associated protein/transcriptional coactivator with PDZ-binding motif) also influence ciliogenesis via regulation of actin polymerisation. Under actin assembly conditions, YAP/TAZ translocates into the nucleus and upregulates ciliary disassembly factors such as Aurora Kinase A (AURKA) and Polo Like Kinase 1 (PLK1), thereby inhibiting ciliogenesis (Das et al., 2016; Kim et al., 2015). Upon actin disassembly, YAP/TAZ is restrained in the cytoplasm and inactivated via F-actin capping proteins such as cofilin, CapZ and gelsolin, inducing ciliogenesis (Aragona et al., 2013; Kim et al., 2015). Notably, these downstream F-actin capping proteins were shown to be inactivated via Lim Domain Kinase 2 (LIMK2) and Testis Associated Actin Remodelling Kinase 1 (TESK1), which represent key players in actin remodelling. LIMK2 and TESK1 promote YAP/TAZ activity, subsequently inducing ciliary disassembly (Kim et al., 2015). An opposing upstream effector of cofilin is the nuclear distribution gene C (NudC), which in contrast to LIMK2/TESK1 stabilises cofilin1, and therefore inhibits ciliogenesis (Zhang et al., 2016).

To make matters more complex, a further study showed that jasplakinolide (Jasp), a potent inducer of actin assembly, actually induced ciliogenesis (Nagai and Mizuno, 2017). This effect is opposite to the studies discussed above which suggest actin polymerisation being antagonistic to ciliogenesis. Jasplakinolide however, was shown to suppress the inhibiting effect of YAP on ciliogenesis (Nagai and Mizuno, 2017). This is a good example of how complex and interconnected actin regulatory drugs affect ciliation.

Further studies indicated that the effect of actin modulators on cilia disassembly might be facilitated through micro-RNAs. Micro-RNA regulation of ciliogenesis has already been discussed in Walentek et al., however, the regulation via actin proteins has not been described (Walentek et al., 2014).

The conserved microRNA *miR-129-3p* was shown to promote both ciliogenesis and ciliary elongation by suppressing the expression of ARP2, the Cdc42 effector TOCA-1, and F-actin binding proteins ABLIM1/3 and CP110 that are all required for branched actin network formation (Cao et al., 2012). The centriolar coiled coil protein of 110 kilodalton (CP110) represents a potent cilia inhibitor since its removal from the mother centriole is necessary for its maturation into a basal body (Reviewed in Tsang and Dynlacht, 2013). In multiciliated cells (MCC), CP110 was also shown to be under control of the microRNA family *miR-34/449* upon coactivation by MCC ciliary transcription factors *rfx2*, *foxj1* and *myb* (Song et al., 2014; Walentek et al., 2016). The same microRNAs *miR-34/449* were found to impact the small GTPase R-Ras and its interactor and actin-binding protein Filamin-A in multiciliated cells (Chevalier et al., 2015; Mercey et al., 2016). Since the apical actin network is required for basal body anchoring, which involves both the actin organising protein R-Ras and Filamin-A, this impaired multiciliogenesis.

In recent years, the role of many actin regulators was investigated in the context of ciliogenesis. It seems that most actin polymerisation factors have a negative role in ciliogenesis potentially via inducing ciliary disassembly factors in complex signalling cascades. On the other hand, actin depolymerisation increases ciliogenesis, which shows that the regulation of actin dynamics has an important effect on ciliogenesis. However, the cilium itself was shown to regulate several signalling pathways which affect downstream actin networks and subsequently ciliogenesis. In the following paragraph we will try to shed light on these complex feedbacks loops while focussing on PCP signalling.

5. Ciliary signalling affecting downstream actin networks

Several actin regulators were shown to impact ciliogenesis in a negative way as described above. On the other hand, many signalling pathways directed by the primary cilium affect downstream actin networks, potentially via interconnections with other networks and subsequently influencing ciliogenesis via feedback loops. Some examples for signalling pathways targeting the actin cytoskeleton are AKT/mTOR, Notch and Hippo signalling (Cao et al., 2017; Seo and Kim, 2018; Wu et al., 2016). One of the ciliary signalling pathways directly acting on the actin cytoskeleton is the non-canonical WNT signalling pathway. It is also referred to as the planar cell polarity (PCP) pathway since its activation results in coordinated orientation of cells within a tissue (Gong et al., 2004; Luo et al., 2020). In contrast to canonical WNT signalling, which is coupled with decreased ciliation, PCP signalling is often accompanied by induced ciliogenesis (Gerdes et al., 2007; Lienkamp et al., 2012; McMurray et al., 2013).

Similar to the canonical/β-catenin dependent signalling cascade, initiation of the PCP pathway requires activation of the Frizzled coreceptor complex via binding of a WNT ligand (Minegishi et al., 2017; Wu et al., 2013). This requires a stable microtubule network upon which Frizzled receptors are trafficked to the membrane to maintain the PCP phenotype (Mathewson et al., 2019). The binding of WNT ligands to Frizzled receptors results in Inversin-mediated recruitment of Dishevelled to the plasma membrane (Simons et al., 2005). Dishevelled further binds to the formin Daam1 and the small GTPase Rac1 which activate downstream signalling cascades via actin regulators RhoA, Rock and Jnk, resulting in rearrangements of the subapical actin network (Habas et al., 2001; Liu et al., 2008). Dishevelled is also regulated via the core PCP protein Vangl2 in both negative and positive ways. Wnt5a activated Vangl2 supports Inversin-mediated recruitment of Dishevelled to the plasma membrane but also inhibits the binding of Dishevelled to Daam1 (Seo et al., 2017; Yang et al., 2017). Vangl2 is one of the core PCP proteins since it interacts with and recruits PCP regulators such as Prickle, affecting downstream RhoA signalling (Nagaoka et al., 2019). Besides influencing the actin network via downstream cascades, both WNT proteins and Frizzled receptors were shown to localise directly to actin dependent cell protrusions such as filopodia and cytonemes, influencing their formation (Mattes et al., 2018; Sagar et al., 2015; Stanganello et al., 2015).

Inversin was one of the first proteins to highlight the association between cilia and PCP signalling. As stated above, upon PCP signalling, Inversin recruits Dishevelled to the plasma membrane where it activates downstream signalling pathways (Simons et al., 2005). Inversin was further found to localise to primary cilia and mutations in the *NPHP2* gene, encoding Inversin, can lead to the ciliopathy nephronophthisis type 2 (Otto et al., 2003; Shiba et al., 2009). Inversin binds to microtubules *in vitro* (Nürnberger et al., 2004) and also impacts the cortical actin network independently of the cell cycle stage (Werner et al., 2013). Mitotic cell rounding, which is dependent on a functional cortical actin

network, is defective in Inversin knockout cells, accompanied by increased filopodia numbers (Werner et al., 2013). It was suggested that the Inversin-dependent actin phenotype might be due to its regulation of Dishevelled, since it was shown that Dishevelled regulates actin via RhoA and Daam1 (Kim and Davidson, 2011; Liu et al., 2008). Transcriptome analysis further revealed dramatic changes in regulators of WNT signalling, focal adhesions and actin dynamics in Inversin knockout cells and mislocalisation of key actin regulators RhoA, Rac1 and Cdc42 accompanied by a PCP phenotype (Veland et al., 2013). Overall, Inversin seems to be a key player in the processes connecting cilia, WNT/PCP signalling and cytoskeletal dynamics.

A proteomic screen identified the formin Daam1, an actin nucleator and interactor of Dishevelled, as a potential component of primary cilia (Ishikawa et al., 2012). Daam1 has been shown to form a complex with the ciliary transition zone protein Nphp4 via the PCP component Intu which regulates the subapical actin network (Yasunaga et al., 2015). Interestingly, Daam1 was further found to localise to ciliary vesicles and its formin/actin binding activity was shown to be required for ciliogenesis in vertebrate kidney cells, although it is not necessary for ciliation in X. laevis embryos (Corkins et al., 2019).

Another protein linking cilia, PCP and actin is the PCP mediator Wdpcp, also known as BBS15. Mutations in the Wdpcp have been found to cause Bardet-Biedl syndrome, featuring cardiac outflow tract and cochlea defects. Similar to Inversin, Wdpcp localises to the transition zone of cilia where it recruits essential ciliary proteins and is further found to regulate actin filaments and focal adhesions (Cui et al., 2013; Kim, Shindo et al., 2010). As one of the key planar cell polarity effector (PPE) proteins, Wdpcp regulates PCP signalling in multiple ways by interaction with Dishevelled and maintaining a stable cortical actin cytoskeleton (Park et al., 2015; Wang et al., 2017).

Highly conserved proteins that regulate PCP and play a role in ciliogenesis, have recently been termed CPLANE proteins (ciliogenesis and planar polarity effectors) (Toriyama et al., 2016). This module contains the classical planar cell polarity effector (PPE) proteins Intu, Fuz and Wdpcp as well as ciliopathy proteins such as Rsg1 and Jbts17 (C5orf42). Further expanding this protein network, the CPLANE interactome consists of IFT-A components, dynein transport proteins and CCT chaperonins. Interestingly, Jbts17 was found to recruit CPLANE to the basal body where it further recruits IFT components. More recent studies identified that CPLANE proteins Intu and Fuz are functionally related to Rab GDP-GTP exchange factors (GEFs), and contribute towards the initial steps of ciliary vesicle docking (Gerondopoulos et al., 2019). Consistent with both studies, knockout of the small GTPase Rsg1, component of CPLANE, was shown to decrease ciliation and result in ciliopathy associated phenotypes including polydactyly (Agbu et al., 2018). For a more detailed review on CPLANE proteins and their function in ciliogenesis see Adler and Wallingford (2017).

The intraflagellar transport (IFT) machinery of the primary cilium is particularly regulated by the BBSome complex (Blacque et al., 2004). Recent data indicate an involvement of both IFT and BBS proteins in maintenance of PCP signalling via trafficking of key PCP regulators. Several Bbs and Ift proteins have been shown to interact with the core PCP protein Vangl2, and knockout of those proteins results in PCP defects in mouse tissues, presumably due to defective Vangl2 trafficking (Jones et al., 2008; May-Simera et al., 2015; McMurray et al., 2013; Ross et al., 2005). In accordance with this, loss of the IFT38 protein Cluap1 leads to enhanced actin stress fibre formation, accompanied by a migration phenotype (Beyer et al., 2018). Additionally, BBS proteins were shown to interact with the PCP protein Inversin and maintain its trafficking to the basal body in ciliated cells (May-Simera et al., 2018, 2015; Patnaik et al., 2019). Besides their association with PCP signalling via Vangl2, several Bbs proteins have further been shown to localise to actin-rich structures such as filopodia or lamellipodia in murine kidney cells, indicating alternative cilia-independent functions for these proteins (Hernandez-Hernandez et al., 2013; Patnaik et al., 2020). They were further shown to affect these structures via downregulation of RhoA levels, which consequently affected ciliogenesis again (Hernandez-Hernandez et al., 2013; May-Simera et al., 2015).

Since PCP affects the polarity of cells via modulation of actin dynamics, it is required for the orientation of basal bodies and primary cilia within epithelial tissues (Boutin et al., 2014; Carvajal-Gonzalez et al., 2016; Fuertes-Alvarez et al., 2018; Namba and Ishihara, 2020). This is especially important for the orientation of motile cilia in respiratory epithelia as well as in sensory hair cells in the developing cochlea, defects of which often accompany the ciliopathy phenotype (May-Simera, 2016; Tsuji et al., 2018; Vladar et al., 2015). The mammalian cochlea represents a good model to study PCP signalling since the elongation and rotation of actin-based hair bundles is highly dependent on correct localisation of the primary cilium, namely the kinocilium. The actin nucleator Cobl (Cordon-Bleu) is crucial for PCM dependent basal body positioning of the kinocilium in the cochlea via affecting actin polymerisation through PCP maintenance (Haag et al., 2018). The Rho GTPase Cdc42 is another PCP regulator which affects kinocilium positioning through interaction with the polarity protein aPKC (Kirjavainen et al., 2015). One of the key PCP proteins required for basal body positioning is Vangl2. Interactions between Myosin 1d and Vangl2 regulate basal body alignment which emphasises an involvement of the actomyosin cytoskeleton in both PCP and ciliogenesis (Juan et al., 2018; Tingler et al., 2018). Vangl2 further recruits Prickle to affect cilia length and basal body positioning (Chu et al., 2016). Another study suggested basal body positioning being dependent on the interaction between vangl2 and arl13b, a key component of the primary cilia membrane and thus affecting PCP signalling in zebrafish (Song et al., 2016).

An interplay between PCP signalling and ciliogenesis could also be shown on the transcriptional level. The transcription factor Foxj1a is required for ciliogenesis in different tissues (Chen et al., 1998; Stubbs et al., 2008). Activity of Foxj1a was already shown to be temporally influenced by the canonical WNT/β-catenin signalling co-transcription factors Lef1 and Tcf7 (Caron et al., 2012; Zhu, Xu et al., 2015). On the other hand, Foxj1a was also shown to be under transcriptional control of the transcription factor TAp73 (p73) (Marshall et al., 2016; Nemajerova et al., 2016), which is required for PCP signalling and actin dynamics via transcriptional modulation of non-muscle Myosin II activity (Fuertes-Alvarez et al., 2018). Hence, Foxj1a seems to be a key regulator of ciliogenesis, which is affected in multiple ways by both β-catenin dependent WNT and PCP signalling.

As seen in this chapter, PCP signalling is regulated by cilia proteins thus affecting actin networks to maintain cell polarity. In Fig. 3, a graphical representation shows the regulation of ciliary signalling on the actin network via key components. However, it is still not clear whether disturbance of ciliogenesis results in PCP defects and consequently actin defects, or if ciliary proteins regulate subsequent actin networks independently of their ciliary role. As described in section 4, actin dynamics regulate ciliogenesis, highlighting the complex feedback loops behind these regulatory mechanisms. The crosstalk between many different effectors and downstream PCP signalling targets highlights the multifactorial regulation between actin dynamics and ciliogenesis.

6. Role of actin in ciliopathies

Defects in cilia function can result in a range of genetic disorders termed ciliopathies. Ciliopathies comprise syndromic diseases such as Bardet-Biedl syndrome, Meckel-Gruber syndrome, Alström syndrome or McKusick–Kaufman syndrome, as well as non-syndromic ciliopathies including retinitis pigmentosa or polycystic kidney disease. Primary phenotypes are characterised by retinal degeneration, hearing loss, obesity, polydactyly and renal defects, with many secondary features occurring including mental retardation or infertility (Goetz and Anderson, 2010; Waters and Beales, 2011). In Fig. 4, some examples of affected tissues are shown with the specific primary cilia depicted. Since actin plays a prominent role in ciliation, many ciliopathy phenotypes are likely attributed to actin-associated mechanisms. In the following

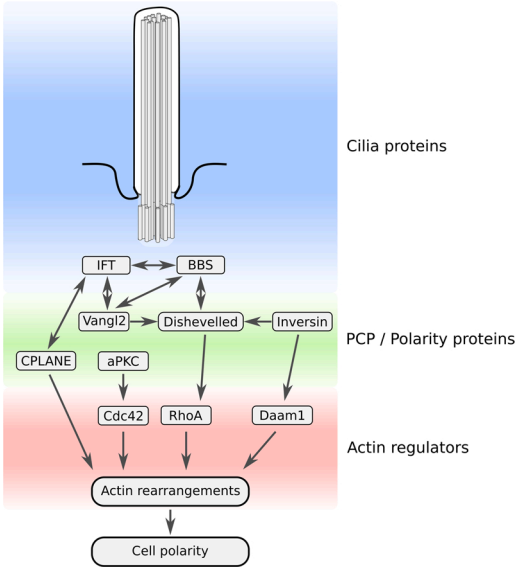

Fig. 3. Ciliary PCP signalling regulates downstream actin dynamics. Cilia IFT and BBS proteins interact with PCP proteins Vangl2, Dishevelled and Inversin. PCP proteins and polarity proteins such as the CPLANE complex activate downstream actin regulators such as Cdc42, RhoA, Daam1, subsequently affecting actin rearrangements and cell polarity. PCP: Planar Cell polarity, IFT: Intraflagellar transport, BBS: Bardet-Biedl syndrome, Vangl2: Van Gogh-Like 2, CPLANE: Ciliogenesis and Planar Polarity Effectors, Cdc42: Cell Division Cycle 42, aPKC: Atypical Protein Kinase C, RhoA: Ras Homolog Family Member A, Daam1: Dishevelled Associated Activator Of Morphogenesis 1.

section, we highlight the role of actin dynamics in ciliary diseases. These newly identified actin-related pathways offer potential entry points into developing therapeutic approaches.

6.1. *Actin function in cilia of the visual system*

Arguably the most common ciliopathy phenotype is retinal degeneration (May-Simera et al., 2017). This is driven by the fact that in addition to being the most common phenotype in syndromic ciliopathies, non-syndromic retinitis pigmentosa genes often encode ciliary proteins and are responsible for roughly 25 % of vision loss in adults (Bujakowska et al., 2017; May-Simera et al., 2017). Mammalian photoreceptor cells are made up of the inner and the outer segment, connected via the so-called connecting cilium (CC). Both the connecting cilium as well as the outer segment represent parts of a highly specialized primary cilium, with the connecting cilium corresponding to the transition zone and the outer segment representing the enlarged ciliary axoneme (Roepman and Wolfrum, 2007, see Fig. 4a). Photoreceptors are required to be highly metabolically active to maintain the constant turnover of membranous discs containing rhodopsin from the inner to the outer segment across the connecting cilium. In the outer segment, these discs are shed and phagocytosed by the retinal pigment epithelium (RPE) (Mazzoni et al., 2014). There are many associations between photoreceptor formation and actin dynamics (also reviewed in Megaw and Hurd, 2018). Early studies showed actin localising to the distal end of the connecting cilia of outer segments and suggested that it might be involved in photoreceptor disc formation during evagination of the plasma membrane at this location (Chaitin et al., 1984; Williams et al., 1988). More recently it was shown that the transport of rhodopsin to the photoreceptor outer segment is mediated by the small GTPase Rab8 in an actin-dependent manner (Deretic et al., 2004). Furthermore, proteomic approaches identified the actin nucleators Arp2/3 as key players in photoreceptor disc formation initiation (Spencer et al., 2019). Studies have shown that loss of numerous actin proteins contributes to photoreceptor degeneration in thus far unclassified diseases (Dollar et al., 2016; Fontainhas and Townes-Anderson, 2011; Moshiri et al., 2017; Ríos et al., 2020; Wang et al., 2019; Wang, Hu et al., 2019; Wang and Townes-Anderson, 2015).

Other aspects of ciliogenesis have also been shown to lead to actin-related retinal degeneration. The protein EYS (Eyes shut homolog) is important for development of photoreceptor cells and mutations of its gene can lead to autosomal recessive retinitis pigmentosa and cone-rod dystrophy (Abd El-Aziz et al., 2008). Knockout studies in zebrafish revealed disrupted F-actin filaments in photoreceptors and defective targeting of outer segment proteins, indicating a role for EYS in F-actin

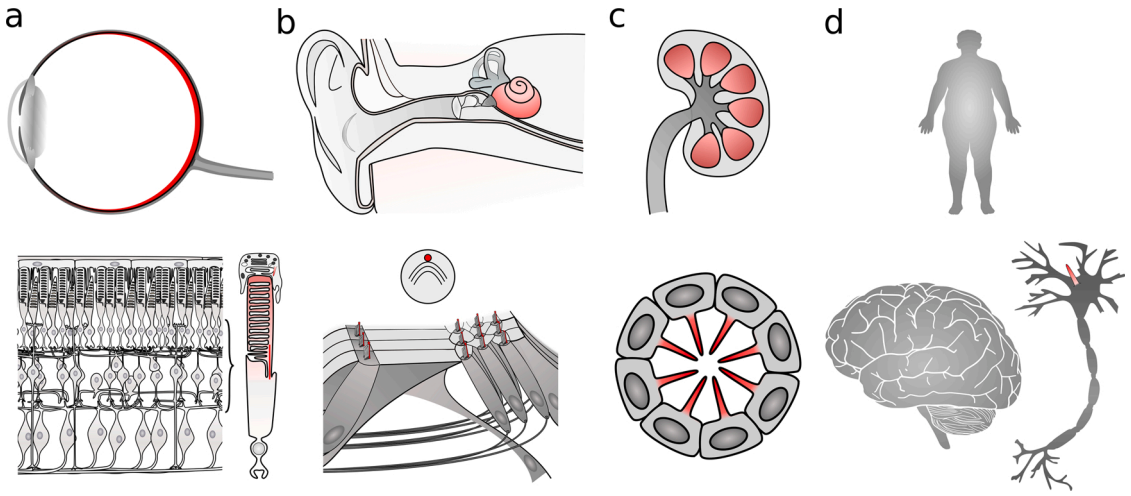

Fig. 4. Primary cilia in tissues affected in ciliopathies. A Primary cilia are essential for visual function in the retina. Both the connecting cilium and the outer segment of the light sensitive photoreceptor neuron represent a highly specialized primary cilium (red). The outer segment attaches to the retinal pigment epithelium (RPE), a pigmented monolayer epithelium with numerous actin-based apical processes, which engulfs shed outer segment discs and is therefore critical for proper photoreceptor function. The RPE is also ciliated and cilia defects affect RPE development and function. **B** In the inner ear, the cochlea epithelium contains hair cells exhibiting actin-based stereocilia, the orientation of which is dependent on a specialized primary cilium, the kinocilium (red). Kinocilia defects result in misaligned stereocilia bundles and are associated with auditory symptoms in patients. **C** The human kidney consists of epithelial cells lining the nephric tubules. Primary cilia are found on the apical side of epithelial cells throughout the renal tubules. Defects in cilia development in the kidney are associated with the ciliopathy polycystic kidney disease (PKD). **D** Hypothalamic neurons also exhibit primary cilia. Ciliary defects in these neurons are thought to be associated with resistance to the satiety hormone leptin, underlying obesity in ciliopathies.

morphology and actin-dependent protein transport into the outer segment (Lu et al., 2017). Further it was shown that the gene retinitis pigmentosa 2 (RP2), mutations of which lead to retinal degeneration, regulates the osteoclast-stimulating factor 1 (OSTF1) as well as Myosin Ie (Myo1e), thereby influencing cell motility in an actin-dependent manner (Lyraki et al., 2018). Mutations in the Retinitis Pigmentosa GTPase regulator (RPGR) leads to X-linked retinitis pigmentosa (Gakovic et al., 2011; Megaw et al., 2017). RPGR has been shown to directly regulate actin disassembly in the photoreceptor connecting cilia via the actin modulator gelsolin, thus causing rhodopsin mislocalisation and blindness in mice (Megaw et al., 2017). In *Rpgr* knockout mouse retinae, actin polymerisation is increased due to upregulation of RhoA and its effector RTKN2 (Rhotekin2), contributing to disrupted ciliary trafficking of M-opsin (Rao et al., 2016). Recent studies attributed this to mutations in Roundabout Guidance Receptor 1 (ROBO1), a known RhoA/actin regulator in axon guidance, which might be regulated by RPGR (Appelbaum et al., 2020). Concordant with this, Patnaik et al. showed upregulated RhoA activity in the absence of Rpgr which was accompanied by increased levels of Dishevelled due to impaired proteasomal degradation (Patnaik et al., 2018). Since Dishevelled mediates PCP signalling subsequently inducing ciliogenesis and actin dynamics, these findings highlight the complex regulation of Rpgr on ciliogenesis via PCP signalling and proteasomal activity.

Mutations in C2orf71/PCARE (photoreceptor cilium actin regulator) lead to inherited retinitis pigmentosa (RP54) characterised by progressive rod photoreceptor loss (Collin et al., 2010; Kevany et al., 2015; Nishimura et al., 2010). C2orf71/PCARE has recently been shown to recruit F-actin to both the basal body and connecting cilia of photoreceptor cells by interacting with the Arp2/3 complex activator WASF3, making it an important regulator of actin-based outer segment disc formation (Corral-serrano et al., 2020). PCARE and several other actin regulators such as Wasf3, cofilin1 and RhoA were further identified in ectosomes shed from murine photoreceptors exhibiting defective disc formation (Spencer et al., 2019).

Photoreceptors are not the only ciliated cell type in visual system. Another critical cell type is the retinal pigment epithelium (RPE) which is required for numerous critical processes in the visual cycle. Actin rich apical processes that extend from the RPE apical membrane engulf photoreceptor outer segments (Fig. 4a), and this interaction is required for correct phagocytosis of shed outer segments. Correct cilia function is vital for development and maturation of the RPE, with ciliary defects resulting in immature apical processes which are unable to phagocytose correctly (Kretschmer et al., 2019; May-Simera et al., 2018; Patnaik et al., 2019). Further studies have shown that F-actin morphology seems to affect phagocytic function through regulation by Rho family GTPases such as Rac1 (Bulloj et al., 2018; Müller et al., 2018), which might also be happening in a cilia-dependent manner. A further effector of actin-dependent phagocytosis is the actin motor protein Myosin VI. Myosin VI enables a rapid, more randomly targeted trafficking of phagosomes to the RPE (Hewage and Altman, 2018). This regulation requires outer segment binding, engulfment and subsequent F-actin assembly and rearrangement beneath bound particles, and seems to incorporate Myosin II as well as Myosin VI, most likely regulated downstream of PI3 kinases (PI3K) and AKT signalling (Bulloj et al., 2013). Finally, two recent transcriptomic studies suggested the activity of the RPE being tightly regulated by actin remodelling of tight junctions in a circadian manner (DeVera and Tosini, 2020; Louer et al., 2020).

6.2. Defects of the auditory system

Besides ocular phenotypes, many ciliopathies are associated with auditory symptoms. One reason for this is that cochlea hair cells in the inner ear display mechanosensing actin-based stereocilia, whose characteristic patterning is dependent on a true microtubule-based kinocilium (Fig. 4b). Virtually all ciliopathy mouse models display misoriented or rotated stereociliary bundles, a readout for defective PCP signalling (Jagger and Forge, 2012; Jones et al., 2008; Montcouquiol et al., 2003; Ross et al., 2005). Kinocilium positioning and disassembly is critical dependent on an intact actin network and PCP signalling, again highlighting the complex interplay between cilia and PCP (Haag et al., 2018; Kirjavainen et al., 2015).

Although the auditory phenotype in most ciliopathies is subclinical, it is highly prominent in Alström syndrome. The basal body protein ALMS1 is not only involved stereocilia organisation (Jagger et al., 2011), but similar to other ciliopathy proteins it also interacts with the actin cross-linker α-actinin 4 (Collin et al., 2012). In line with this, ALMS1 fibroblasts show abnormalities in actin stress fibre morphology (Collin et al., 2012).

6.3. Actin in kidney disease development

One of the most critical clinical features affecting ciliopathy patients is kidney disease (Devlin and Sayer, 2019). So far, several studies have shown that actin disorganisations contributes to ciliopathy-related kidney diseases. Fig. 4C depicts a nephric tubule with the primary cilia oriented to the apical side. A recent proteomic study revealed downregulations of actin-related proteins in urinary extracellular vesicles of ciliopathy patients exhibiting kidney phenotypes (Stokman et al., 2019).

Among inherited cystic kidney disorders, autosomal dominant polycystic kidney disease (ADPKD) is the most common form, and it is caused by mutations in polycystic kidney disease (PKD) 1 or 2 (Torres and Harris, 2009). Polycystin-1 and 2 (PC1/2), the proteins encoded by the PDK genes, form a G-protein coupled receptor complexed with a calcium permeable channel. Both PC proteins were shown to localise to primary cilia in the kidney (Pazour et al., 2002; Yoder et al., 2002) and they are involved in the calcium signalling branch of WNT signalling (Kim et al., 2016). However similar to other PCP proteins, PC1 was further shown to localise to other cellular regions and to affect actin dynamics. In particularly, PC1 is found at lamellipodia of migrating kidney epithelia cells where it forms a protein complex with the key actin regulator N-Wasp and Pacsin-2 (Yao et al., 2014). Moreover, PC1 is required for proper binding of N-Wasp to Arp3, thus consequently affecting actin nucleation and lamellipodia formation. In a more recent approach, miRNA profiles of PKD mouse models revealed an upregulation for miRNA-182-5p, which targets key actin target genes such as Wasf2, Dock1, and Itga4 (Woo et al., 2017). This resulted in inhibited actin cytoskeleton formation and increased cyst formation. Finally, PC1 was recently shown to regulate RhoA and ROCK signalling via interaction with the specific RhoGAP ARHGAP35, subsequently affecting cilia length (Streets et al., 2020).

Besides PC1, several studies focused on its complex partner PC2, a calcium^{2+} permeable ion channel. PC2 was also shown to localise to lamellipodia and possibly associates with the actin cytoskeleton via cortractin (Gallagher et al., 2000). PC2 directly interacts with the actin bundling protein alpha-actinin in several kidney cell lines and its calcium channel activity was shown to be influenced by several actin-binding proteins like alpha-actinin, filamin, profilin and gelsolin (Cantero and Cantiello, 2015; Li et al., 2005; Wang et al., 2015). It was further shown to interact with the RhoA GTPase binding formin mDia1 and to act directly on actin via filamin-A in a calcium-dependent way (Rundle et al., 2004; Wang et al., 2015).

A third ciliopathy protein highlighting the connection between actin and cilia is the inositol polyphosphate 5-phosphatase OCRL1, mutations in which cause the kidney phenotype in Lowe syndrome. The OCRL1 protein localises to primary cilia in kidney cells and affects ciliary length when mutated (Coon et al., 2012; Luo et al., 2012; Rbaibi et al., 2012). Even before Lowe syndrome was diagnosed as a ciliopathy, OCRL1 deficiency was shown to affect actin architectures and regulators like gelsolin and alpha-actinin (Suchy and Nussbaum, 2002). OCRL1 maintains RhoA-Cofilin signalling via activation of Rac1 during membrane remodelling (Coon et al., 2009; Madhivanan et al., 2012; Vicinanza et al., 2011; Zhu et al., 2015).

6.4. Actin regulation of obesity

Another hallmark ciliopathy phenotype is obesity possibly caused by defective cilia on hypothalamic neurons (Davenport et al., 2007). This phenotype is accompanied by a deficiency or resistance to the physiological satiety hormone leptin (Han et al., 2014). Treatment with leptin resembles actin depolymerisation through cytochalasin D and increases ciliary length in hypothalamic neuronal cells (Kang et al., 2015). Leptin inhibits PTEN/GSK3β signalling which results in upregulation of anterograde IFT protein mRNA. Since PTEN directly regulates the actin depolymerising factor cofilin-1 and GSK3β controls actin dynamics via Rac and Rho GTPases (Serezani et al., 2012; Sun et al., 2009), leptin seems to regulate ciliogenesis both via transcriptional activation of cilia genes and upstream regulation of actin rearrangements (Kang et al., 2015). Thus, Kang et al. suggested that F-actin depolymerisation is an important downstream signalling pathway involved in leptin regulation of ciliary assembly. Knockout of the ciliopathy protein Lztfl1, also known as BBS17, leads to leptin resistance and cilia deficiency in mice (Seo et al., 2009; Wei et al., 2018). Interestingly, Lztfl1 was found to directly interact with actin and actin-binding proteins, thus the authors suggested that Lztfl1 is involved in downstream leptin signalling via actin dynamics (Wei et al., 2018).

7. Actin and cilia in cancer

The role of actin in cancer progression has been known for a while since remodelling of the actin cytoskeleton is indispensable for cancer invasion and metastasis (Yamazaki et al., 2005). In recent years, researchers started seeing both a positive and negative impact of primary cilia on cancer progression highlighting this complex relationship (Sarkisian and Semple-Rowland, 2019). For example, in anaplastic ependymomas as well as choroid plexus carcinomas, ciliogenesis is decreased due to downregulation of the transcription factor FOXJ1 (Abedalthagafi et al., 2016). In medulloblastoma or glioblastoma, primary cilia were found to both initiate and inhibit tumour formation, in close relation to their regulation of Hedgehog signalling (Han et al., 2009; Hoang-Minh et al., 2016; Wong et al., 2009). More recently, the role of actin in cancer-related ciliogenesis came into focus, bringing both fields together. Interestingly, cilia in glioblastoma performed exocytosis at the ciliary tip coinciding with F-actin staining at that location (Hoang-Minh et al., 2018). This suggests an involvement of actin in the process of ciliary shedding similar to the processes already described in non-cancerous cell lines (Nager et al., 2017; Phua et al., 2017; Wang, Hu et al., 2019).

One protein known to link ciliogenesis and cancer is the Clusterin Associated Protein 1 (Cluap1/IFT38). Cluap1 is an integral component of the IFT complex of the primary cilium and is highly upregulated in colon cancer (Takahashi et al., 2004). More recently, it was shown that loss of Cluap1 also leads to enhanced actin stress fibre formation accompanied by a migration phenotype (Beyer et al., 2018). The small peptide Thymosin β-4 sequesters G-actin and enhances actin remodelling (Kim and Jung, 2016), subsequently increasing metastatic properties of several cancer cell lines (Cha et al., 2003; Kobayashi et al., 2002; Lee et al., 2015). In human cervical cancer cells, Thymosin β-4 was found to interact with the cilia protein NPHP3, stabilising ciliogenesis (Lee et al., 2019). In that context, Lee et al. suggested that Thymosin β-4 acted on ciliogenesis via regulation of actin stress fibres. In another study, the known tumour suppressor and actin binding protein EPLIN (Epithelial Protein Lost In Neoplasm) was found to be a negative regulator of ciliogenesis and cilia length via stabilisation of actin and translocation of Myosin Va (Gonçalves et al., 2020). Another tumour suppressor, the tumour protein Tp73, regulates PCP signalling via remodelling of the actin cytoskeleton and therefore affects the unidirectional orientation of motile cilia in multiciliated tissues (Fuertes-Alvarez et al., 2018).

Although ciliopathies do not have a higher incidence for cancer, many studies correlate the progression of cancer with defects or enhancements of ciliation, suggesting a critical balance between ciliation and cancer. Just as actin plays a role in both ciliogenesis and cancer, newer research combines both approaches and shows that perturbed ciliary signalling might contribute to the development of certain cancers. However, as discussed before, it is still not clear if defects in ciliogenesis affect downstream actin networks subsequently enhancing tumour growth, or if a remodelling of actin dynamics results in defective ciliogenesis which in turn affects cancer progression.

8. Conclusions

Ciliopathy patients exhibit several clinical features such as retinal degeneration, cochlea and renal defects and obesity. Besides these common phenotypes, other symptoms include mental deficits or brain disorders, polydactyly or liver and heart defects. Although ciliopathies do not have a higher incidence for cancer development, many studies also see correlations between the level of ciliation and tumour progression. Several studies indicate defective actin dynamics as underlying cause for defects in ciliogenesis. The emerging field highlighting the interaction between cilia and actin regulators might help to clarify the molecular mechanisms underlying several ciliopathies. However, it is still not clear why so many cilia proteins localise to and regulate actin networks. Is this regulation independent of their ciliary function or do they exert the same function at different locations? This review depicted different regulatory pathways such as actin dynamics regulating ciliogenesis, however ciliogenesis itself impacts actin networks via downstream signalling pathways. Since these complex feedback loops are often cell type specific, it is vital to uncover the precise molecular mechanisms underlying ciliogenesis and actin dynamics to identify possible starting points for therapeutic approaches in the future.

Declaration of Competing Interest

None.

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3. Discussion

To understand the molecular mechanisms underlying human ciliopathies such as Bardet-Biedl syndrome (BBS), it is imperative to know both the ciliary and non-ciliary functions of ciliary proteins. Besides their well-described role during ciliogenesis, BBS proteins have been found to affect cilia-related Wnt signalling. Recent data also indicate alternative functions of BBS proteins in intracellular trafficking, cell cycle regulation, proteasomal degradation, DNA damage response, transcriptional regulation, and the actin cytoskeleton. In the context of this thesis, the cilia-dependent and independent functions of BBS proteins were investigated in more detail with a particular focus on their role during Wnt signalling. Thus, *Bbs* gene levels in different mouse tissues were analysed to identify potential tissue-dependent functions of BBS proteins. Since one of the primary features of Bardet-Biedl syndrome is vision loss, the function of BBS proteins during development of the retinal pigment epithelium (RPE) was investigated in more detail and set in context to cilia related Wnt signalling. And finally, since Wnt signalling affects the downstream actin networks, the regulation of the actin cytoskeleton via BBS proteins was of further interest. The following sections describe and discuss the findings of the presented publications and manuscripts that make up this thesis and the additional data incorporated in section 3.3.

3.1. Investigation of potential tissue-dependent regulations of BBS proteins

Bardet-Biedl syndrome is a pleiotropic and variable ciliopathy (Badano et al., 2006; Katsanis, 2004), which can affect various organs and tissues, such as the retina, kidney, genitals, bone or brain (Beales et al., 1999; Florea et al., 2021; Forsythe and Beales, 2013). There is no distinct genotype-to-phenotype correlation, further complicating the diagnosis (Daniels et al., 2012; Deveault et al., 2011). Factors that affect the phenotypic occurrence of BBS are genetic heterogeneity, gene modifiers and the involvement of BBS proteins in various protein networks and signalling pathways that together have diverse symptomatic effects (Florea et al., 2021). On the other hand, mutations in a few select *BBS* genes can still be associated with a specific phenotype. For example mutations in the genes encoding the three chaperonin-like proteins BBS6, BBS10 and BBS12 often show overlapping phenotypes that are often more severe than mutations in other *BBS* genes (Billingsley et al., 2010; Castro-Sánchez et al., 2015; Imhoff et al., 2011). Moreover, mutations in *BBS6, BBS10* or *BBS12* are more often associated with

kidney phenotypes, whereas mutations in the *BBSome* genes might correlate with hypogonadism (Imhoff et al., 2011; Manara et al., 2019). The BBSome core components BBS2, BBS7 and BBS9 are furthermore often implicated in kidney development and health (Niederlova et al., 2019). **Publication I** aimed to identify possible correlations between *Bbs* gene expressions in different mouse tissues and their known protein function in relation to symptomatic features.

3.1.1. Tissue-dependent differences in expression of the BBSome subunits

To investigate the potential tissue dependent functions of BBS proteins in more detail, the expression profile of *BBSome* genes was assessed in different mouse organs including brain, kidney, lung, spleen, heart, oviduct, and retina (**Publication I**). This analysis revealed that the expression levels of most *BBSome* components (with the exception of *Bbs1* and *Bbs18*) was significantly upregulated in the retina in comparison to other tissues. Since one of the main features of Bardet-Biedl syndrome is blindness, the high expression of *BBSome* components suggests a prominent role for these proteins within the retina. Indeed, photoreceptor cells contain a highly specialised primary cilium comprising the connecting cilium between inner and outer segment and the outer segment (May-Simera et al., 2017). The BBSome is required for efficient trafficking across the connecting cilium, highlighting its importance within the retina (Bales et al., 2020; Datta et al., 2015). Since the photoreceptor outer segment is constantly renewed and has a high protein turnover, the role of the BBSome in facilitating cargo trafficking within outer segments is critical. Some BBSome components such as BBS5 or BBS8 have retina-specific isoforms, which might be the consequence of the important role of the BBSome during retinal ciliary transport (Bolch et al., 2016; Riazuddin et al., 2010). In contrast to cilia in other tissues, the BBSome within photoreceptor cells can enter the cilium even when it is not completely assembled; only BBS8 is required for the entry of the (partial assembled) BBSome into photoreceptor cilia, highlighting early on a tissue-specific ciliary function (Dilan et al., 2018; Hsu et al., 2021).

Knockout of *Bbs8* in mouse tissues led to a significant downregulation of several components of the *BBSome*, of which the BBSome core component *Bbs7* was most strongly affected (**Publication I**). Particular in the retina, most BBSome components were significantly downregulated upon loss of *Bbs8*, underlining the importance of Bbs8 in photoreceptor cilia (Dilan et al., 2018; Hsu et al., 2021). Loss of the chaperonin-like protein Bbs6, which is required

for BBSome assembly, did not affect the expression of *BBSome* components in most tissues remarkably. However, all *BBSome* components except *Bbs2* were notably upregulated in spleen, suggesting that the interaction between Bbs6 and the BBSome in spleen is highly critical for BBSome function.

3.1.2. Tissue-dependent differences in expression of the chaperonin-like Bbs genes

In comparison to the BBSome, expression levels of the three chaperonin-like *Bbs* genes did not vary a lot between different tissues (**Publication I**). However, it was conspicuous that *Bbs12* was slightly more abundant in spleen, oviduct and retina tissues. Although all three chaperonin-like proteins carry several insertions which make them non-functional chaperonins, it is feasible that BBS12 is more similar to CCT chaperonins and thus has a more important function in different tissues (Kim et al., 2005; Mukherjee et al., 2010; Stoetzel et al., 2007, 2006). It is also plausible that it might be required to fold other proteins besides the BBSome in a tissue-dependent mechanism. In regard to the phenotypic occurrence of BBS, mutations of the three chaperonin-like genes are often associated with a kidney phenotype, however an increased expression of these genes in the kidney was not observed (Imhoff et al., 2011; Manara et al., 2019).

Loss of *Bbs8* also affected the levels of the chaperonin-like genes in different tissues, however the effect was not as significant as for some of the BBSome components. Since the chaperonin-like complex is required for BBSome assembly, the observed changes of *Bbs6*, *Bbs10* or *Bbs12* upon loss of *Bbs8* might be due to transcriptional feedback regulations. Interestingly, in the oviduct, *Bbs12* compensating the reduced expression of *Bbs6* due to loss of *Bbs8* could be observed, suggesting an important role for Bbs8 in the oviduct. Concordantly, mutations in *BBSome* components are more often associated with hypogonadism (Imhoff et al., 2011; Manara et al., 2019). Surprisingly, loss of *Bbs6* mostly left expression levels of *Bbs10* and *Bbs12* unaffected. Since BBS6 and BBS12 together recruit BBS7 to enable its folding by the CCT chaperonin complex, it is plausible that Bbs12 can compensate the loss of *Bbs6* in most tissues on a functional but not transcriptional level, which might be why an increased expression of *Bbs12* upon loss of *Bbs6* was not observed (Seo et al., 2010; Zhang et al., 2012). Still, a tissue-dependent function for Bbs6 and the chaperonin-like complex could be suggested as most BBSome components were significantly upregulated in spleen upon loss of *Bbs6*. However,

there is only one case report of BBS coinciding with splenic lobulations (Doneray and Orbak, 2006). *Bbs10* as part of the chaperonin-like complex was also significantly upregulated in spleen. Interestingly, *Bbs12* was the only gene not affected by loss of *Bbs6* in spleen, indicating a potential chaperonin-independent function of *Bbs12* in this tissue.

Taken together, a differential expression of *BBS* genes among various tissues was observed, strongly indicating tissue-specific functions of BBS proteins. Especially for the *BBSome* genes, a higher variability between tissues was observed than for the chaperonin-like complex. *BBSome* genes were higher expressed especially in the retina, underlining the important function of the BBSome for photoreceptor ciliary transport. However, the higher prevalence of some BBSome components such as *Bbs5* and *Bbs18* in oviduct or *Bbs1* in spleen, heart and oviduct suggests specific tissue-related functions for these proteins that might be independent of their BBSome function. Regarding the chaperonin-like complex, is could be suggested that especially Bbs12 might exert a non-BBSome-related folding function since it was highly expressed in spleen and not affected by loss of *Bbs6* in this tissue. Although mutations in the chaperonin-like *Bbs* genes are often associated with kidney phenotypes (Imhoff et al., 2011; Manara et al., 2019), a higher expression of these genes in the kidney was not observed.

3.1.3. Conclusion and outlook

The analysis of tissue-dependent differences in gene regulation of components of the BBSome and the chaperonin-like complex provides a better understanding of potential genotype-to-phenotype correlations. For example a higher expression of *BBSome* components in the retina is concomitant with the important function of the BBSome during photoreceptor transport (Bales et al., 2020; Datta et al., 2015). Although kidney symptoms are one of the most commonly occurring features of Bardet-Biedl syndrome, gene levels of the *Bbs* genes were not strikingly affected in this tissue. However, it has to be taken into account, that only mRNA levels of the *Bbs* genes were analysed which might be not related to the protein level or function at all. Higher mRNA expression of distinct genes does not necessarily lead to higher protein expression or compensation of its function.

Thus, it can be concluded that more factors might be involved in the regulation of protein functions. It has to be considered that splicing occurs after transcription affecting protein level and function, which was not taken into account in **Publication I**. Splicing factors have been implicated in ciliopathy symptoms such as retinitis pigmentosa (Maxwell et al., 2021) and cilia

proteins were shown to be involved in splicing and transcriptional regulation (Gascue et al., 2012; Yildirim et al., 2021). Further studies show that RNA modifications can impact cilia protein translation and provoke or modify ciliary phenotypes (Haward et al., 2021; Li et al., 2021; Lin et al., 2018). On the other hand, BBS proteins such as BBS6 and BBS7 were also found to be involved in transcriptional regulation (Gascue et al., 2012; Scott et al., 2017), suggesting feedback loops to regulate their protein level and function. RNA sequence analysis followed by mass spectrometry of *BBS* genes and proteins would be needed to identify the consequence of splicing factors on BBS protein expression and function.

Furthermore, no distinct genotype-to-phenotype correlation suggests the involvement of other unknown genes and proteins in the development of human ciliopathies. Mutations in genes that were beforehand thought to be unrelated to cilia might result in ciliopathy symptoms, leading to their identification as ciliary genes. One example might be provided by **Manuscript I**, which will be discussed below, where a ciliary phenotype was caused by loss of the filopodia regulator Fascin-1, a protein not implicated with ciliopathies beforehand. In case of Bardet-Biedl syndrome, a ciliopathy that has been studied for many years, the most recent BBS proteins were only recently identified, demonstrating the highly dynamic development of this field of research (Morisada et al., 2020; Wormser et al., 2019). BBS patients were often found to carry additional mutations in other ciliopathy genes, explaining overlaps between ciliopathies but also providing the potential for more undiagnosed mutations that were not screened for (Lindstrand et al., 2016; Qi et al., 2017). For the diagnosis of Bardet-Biedl syndrome and a distinct genotype-to-phenotype correlation, a whole-genome sequencing is needed to exclude potential additional mutations in other genes that might affect the phenotype. However, the studies described in **Publication I** provide a valuable insight in the importance of selected *Bbs* genes in different tissues. Detailed analysis of protein levels via mass spectrometry or western blotting will help to clarify the correlation between gene and protein levels, whereas histology of isolated tissue will help to understand potential alternative functions of Bbs proteins in distinct tissues.

3.2. Involvement of ciliary BBS proteins in Wnt signalling

Primary cilia are involved in various aspects of tissue development. They are required for the communication between cells as they send out and receive signals. This also involves intracellular signal transduction, resulting in the regulation of several signalling pathways such as Wnt, Sonic hedgehog (Shh), Platelet-derived growth factor (PDGF), Notch, Transforming growth factor β (TGFβ) or mammalian Target of rapamycin (mTOR) (Anvarian et al., 2019; Lai and Jiang, 2020; Lee, 2020; Li et al., 2020). Primary cilia and cilia-related Wnt signalling are especially important for the development of the retinal pigment epithelium (RPE) (May-Simera et al., 2018; Schneider et al., 2021; Sun et al., 2021). The RPE is a monolayer of cells between retina and choroid, tightly attached to the photoreceptor outer segments and crucial for photoreceptor function. To prevent damage by photo-oxidative stress, photoreceptors constantly regenerate disks within the outer segment. The shed disks are then phagocytosed by the adjacent RPE cells (Mazzoni et al., 2014). Dysfunction of the RPE affects photoreceptor health which can consequently result in the development of retinopathies (Caceres and Rodriguez-Boulan, 2020). Thus, the function of primary cilia during RPE development is of further interest to understand the underlying mechanisms of retinal degeneration and vision impairment in ciliopathies.

As shown in **Publication II**, RPE development and maturation is tightly linked to ciliary assembly and disassembly. Number and length of primary cilia increases during early RPE maturation until ciliary disassembly is initiated upon RPE maturation. Concomitantly, loss of the ciliary genes *Bbs6* and *Bbs8* was shown to disturb RPE maturation and function, terminally resulting in retina degeneration and vision loss in mice (Kretschmer et al., 2019; Schneider et al., 2021). Since loss of the BBS proteins BBS6 and BBS8 in hTERT-RPE1 cells reduced cilia numbers and length in **Publication II**, these data suggest that BBS6 and BBS8 might be involved in the fine regulation of ciliary disassembly processes. This might be coordinated via two processes that are tightly interconnected and will be discussed in more detail below: 1. disassembly factors such as HDAC6 (histone deacetylase 6), that destabilises ciliary tubulin resulting in cilia disassembly, and 2. regulation of the Wnt signalling pathway (section 1.3.). Briefly, during canonical Wnt signalling, binding of the WNT ligand to the Frizzled-LRP5/6 signalosome leads to the recruitment of DVL, CK1α, GSK3β and Axin, subsequently inhibiting the β-catenin destruction complex (Cong et al., 2004; Cselenyi et al., 2008; Davidson et al., 2005; Krasnow et al., 1995; Piao et al., 2008; Stamos et al., 2014). β-catenin accumulates and enters the nucleus, where it activates the transcription of Wnt target genes (Fig. 4). During

non-canonical Wnt signalling, binding of the WNT ligand results in Inversin recruiting DVL, thus activating signalling cascades that target the downstream actin network (Habas et al., 2001; Liu et al., 2008; Simons et al., 2005). Without inhibition, the β-catenin destruction complex phosphorylates β-catenin, resulting in its proteasome-dependent degradation.

3.2.1. BBS proteins interact with Inversin in regulating Wnt signalling

There is already lots of evidence for the involvement of BBS proteins in Wnt signalling. In zebrafish, loss of *bbs6* and *bbs8* leads to a PCP phenotype which is accompanied by increased canonical Wnt activity (Gerdes et al., 2007; May-Simera et al., 2010). Polarisation of stereocilia hair bundles in the developing cochlea is lost upon loss of *Bbs6* and *Bbs8*, resembling a classical PCP phenotype (May-Simera et al., 2015; Ross et al., 2005). BBS8 was further found to interact with both PCP effector proteins Vangl2 and Inversin (May-Simera et al., 2018, 2015, 2010). Concordantly, BBS6 was also found to interact with Inversin (**Publication II**). Since Inversin localisation at the primary cilium was reduced upon loss of *Bbs6*, it is feasible that Bbs6 is needed to recruit Inversin to the primary cilium where it activates the switch from canonical to non-canonical Wnt signalling.

Besides Inversin regulating Wnt signalling via recruitment of DVL (Simons et al., 2005), it further interacts with and inhibits Aurora A kinase (AurA) (Mergen et al., 2013). Without inhibition, AurA interacts with HEF1 (human enhancer of filamentation 1), phosphorylating and activating the ciliary disassembly component HDAC6 (Fig. 5) (Pugacheva et al., 2007). Thus, it was suggested that BBS proteins, as a consequence of PCP signalling, stabilise primary cilia via interaction with Inversin, thus inactivating AurA and HDAC6-mediated disassembly.

3.2.2. BBS proteins affect Wnt signalling via regulation of β-catenin levels

Additionally, HDAC6 deacetylates and stabilises the Wnt signalling effector β-catenin, resulting in the activation of canonical Wnt signalling (Li et al., 2008). Via interaction with Inversin and subsequent regulation of AuroraA-HDAC6, BBS6 and BBS8 were found to promote non-canonical Wnt signalling via precise regulation of β-catenin phosphorylation and acetylation levels (**Publication II**). During non-canonical Wnt signalling, β-catenin is first phosphorylated at residue Ser45 by CK1α which initiates its phosphorylation at Ser33, Ser37 at Thr41 by GSK3β (Amit et al., 2002; Ikeda et al., 1998; Liu et al., 2002; Wu and He, 2006).

Phosphorylation of β-catenin then enables its ubiquitin-dependent proteasomal degradation (Hart et al., 1999; Yanagawa et al., 2002; Yost et al., 1996). On the other hand, the role of acetylation of β-catenin at Lys49 via CBP is controversial and seems to be cell-type specific. Several studies suggest that acetylation of β-catenin is required for its transcriptional activation of Wnt target genes and is thus associated with canonical Wnt signalling (Chen et al., 2020; Hoffmeyer et al., 2017; Iaconelli et al., 2015; Liu et al., 2020; Wang et al., 2013; Yang et al., 2008). On the other hand, the acetylation of β-catenin is also suggested to enable its phosphorylation and subsequent proteasomal degradation (Li et al., 2008; Schofield et al., 2013; Wolf et al., 2002). As mentioned above, the acetylation of β-catenin seems to be rather cell-type and promoter specific and is finely coordinated by different acetylases and deacetylases. As shown in **Publication II**, in hTERT-RPE1 cells, deacetylation of β-catenin via HDAC6 is associated with its active, non-phosphorylated form, thus activating canonical Wnt signalling.

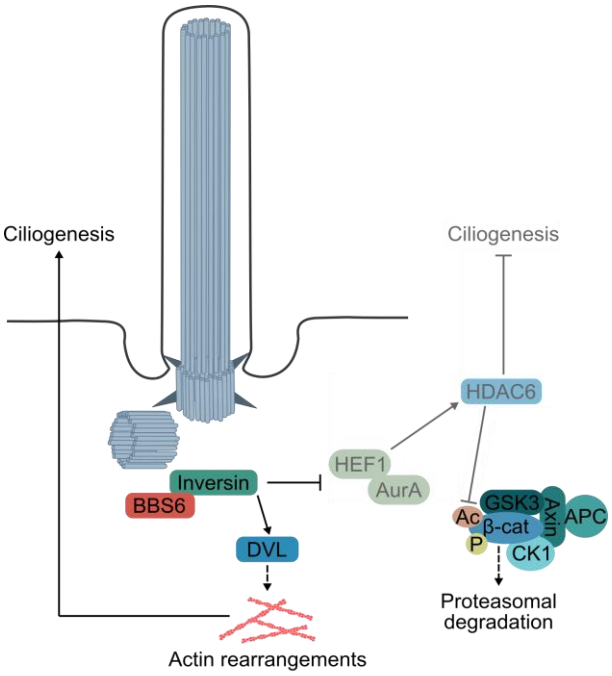

Fig. 5: BBS proteins affect ciliogenesis via regulation of Inversin.
BBS6 is required to recruit Inversin to the primary cilium where it interacts with and inhibits HEF1. Thus, HEF1 is not able to activate HDAC6 together with AurA, so that HDAC6 cannot induce ciliary disassembly by deacetylation of ciliary tubulin. Without HDAC6 activated, β-catenin is acetylated and phosphorylated, consequently targeted for proteasomal degradation. On the other hand, Inversin activates non-canonical Wnt signalling via interaction with Dishevelled (DVL) at the ciliary base, which results in the activation of downstream signalling cascades, activating the downstream actin network. Since a stable actin network is needed to induce ciliogenesis, this might create a feedback mechanism in stabilising primary cilia. Ac: Acetylation; P: phosphorylation.

In conclusion, a two-way mechanism was identified during which BBS proteins protect primary cilia from disassembly via interaction and recruitment of Inversin. Since this affects post-translational modifications of β-catenin, BBS proteins promote non-canonical Wnt signalling as well, which is in line with previous data (Fig. 5) (Gerdes et al., 2007; May-Simera et al., 2018, 2015, 2010; Ross et al., 2005).

However, the mechanism behind this is not completely solved since loss of Inversin generates a cell-type specific ciliary phenotype, not always correlating with reduced cilia numbers and length (Mergen et al., 2013; Veland et al., 2013). The same seems to be true for BBS6 since there are inconsistent data on the ciliation phenotype in *Bbs6* deficient kidney medullary cells (Hernandez-Hernandez et al., 2013; Volz et al., 2021).

A potential explanation for the complex and tissue-dependent differences observed might be ascribed to the suppression of canonical Wnt signalling. Ciliogenesis is associated with non-canonical Wnt signalling as described above (Balmer et al., 2015; Corbit et al., 2008; Cui et al., 2013; Gerdes et al., 2007; May-Simera et al., 2015, 2010; McMurray et al., 2013; Wang et al., 2017), however conversely, downstream rearrangements of the actin network as a result of non-canonical Wnt could also affect ciliogenesis in complex feedback mechanisms as described in section 1.1.2 and **Publication III**. Taken together, these data only shed light on a small part of the big picture including ciliogenesis, Wnt signalling, ciliary disassembly components and the actin network (Fig. 5). More insight into the co-regulation between BBS proteins, cilia and actin will be provided in section 3.3.

3.2.3. Conclusion and outlook

Publication II provides a detailed analysis of how BBS proteins regulate ciliary Wnt signalling via Inversin and HDAC6 and are involved in ciliary disassembly. The analysis of the pathways underlying ciliopathies provides a starting point for the development of therapeutic approaches that target these pathways. For example, in polycystic kidney disease (PKD), a common phenotype of ciliopathies, therapeutic approaches targeting Wnt and HDAC6-related signalling have demonstrated promising results since *PKD1* and *PKD2*, the responsible genes mutated in polycystic kidney disease, are known Wnt cell-surface receptors. Pharmacological inhibition of HDAC6, affecting both ciliogenesis and Wnt signalling as discussed above, has been shown to reduce the formation of kidney cysts and kidney failure in mouse models of polycystic kidney disease, showing its potential for clinical applications (Li, 2011). Concordantly, direct

inhibition of β-catenin improved renal function and decreased lethality of *Pkd* mouse models (Li et al., 2018). In relation to ciliogenesis, inhibition of canonical Wnt signalling in iPSC-derived RPE cells restored cilia and maturation of the RPE (May-Simera et al., 2018). Treatment with the Wnt antagonist Dkk1 rescued differentiation of osteoblasts and abnormal cilia formation previously induced by coculture with gastric cancer cells (Xu et al., 2021). Thus, the data of **Publication II** elucidate the regulations between primary cilia, BBS proteins and Wnt signalling in more detail, providing a basis for understanding the underlying mechanisms and how Wnt inhibitors in therapeutic approaches would affect cell homeostasis on a broader background.

However, it has to be taken into account that several pathways might interact in regulating cell homeostasis and ciliogenesis as shown by the example of HDAC6 that regulates deacetylation of both ciliary tubulin and β-catenin. Especially downstream Wnt targets are often regulated via other processes as well, such as Cyclin D1 as a prominent cell cycle target. β-catenin and Inversin are both phosphorylated via Akt, part of the mTOR pathway, showing complicated phosphorylation regulations that might affect each other subsequently (Ponce et al., 2011; Suizu et al., 2016). Furthermore, the acetylation of β-catenin was shown to be highly tissue-dependent (Chen et al., 2020; Li et al., 2008; Liu et al., 2020; Schofield et al., 2013; Wolf et al., 2002), complicating the understanding of these pathways in different tissues.

The discussion of the data of **Publication II** is moreover based on the finding that ciliogenesis is accompanied by inhibited canonical Wnt and increased PCP signalling (Gerdes et al., 2007; May-Simera, 2016; May-Simera et al., 2010; May-Simera and Kelley, 2012; Simons et al., 2005; Volz et al., 2021). Contrary, it was suggested that Wnt signalling in total is not correlating with ciliogenesis at all (Bernatik et al., 2021), supported by data showing that specific Wnt ligands (WNT3a/WNT5a) can induce YAP/TAZ signalling independently of Wnt signalling (Park et al., 2015). However, when analysing these data, it must be considered that PCP signalling in cells does not resemble a tissue phenotype, where the regulation of PCP is even more critical, for example in epithelial polarisation. Taken together, these data again indicate tissue-dependent differences in the regulation of Wnt signalling which might be of further interest for the studies in **Publication I**, where the analysis of selected *Wnt* genes in different tissues might be useful. A more detailed analysis of the downstream Wnt phenotypes of different tissues lacking *Bbs6* might be of further interest to gain a better understanding of the regulation between Bbs proteins and Wnt.

In conclusion, **Publication II** provides a valuable insight into how BBS proteins affect ciliary disassembly and Wnt signalling, although the exact cooperation between Wnt and ciliogenesis still needs further investigation. Since Wnt signalling targets the downstream actin cytoskeleton, the interplay between BBS proteins, Wnt and actin will be discussed in the following section.

3.3. Interplay between primary cilia and the actin network

Primary cilia are involved in the regulation of several signalling pathways such as Wnt, Sonic hedgehog (Shh) or Platelet-derived growth factor (PDGF) (Anvarian et al., 2019; Bershteyn et al., 2010; Lee, 2020). The regulation of Wnt signalling coordinates various cellular processes including differentiation, proliferation, polarisation and migration. Many of these processes are maintained via changes of the subapical actin network. Especially non-canonical Wnt (PCP) signalling results in actin polymerisation and cell migration because it targets many important actin regulators such as formins and Rho GTPases. Since BBS proteins promote PCP signalling via regulation of Inversin (**Publication II**), it is feasible that they also affect the downstream actin network. There is already lots of evidence for BBS proteins in regulating actin and actin-related proteins either as a downstream response of PCP signalling or as a possible alternative non-ciliary function of BBS proteins. The Bbs proteins Bbs8 and Bbs9 were found to localise to actin-based focal adhesions, indicating a regulatory role at these sites (Hernandez-Hernandez et al., 2013). Bbs4, Bbs6 and Bbs8 organise the apical actin network and inhibit polymerisation of actin stress fibres via regulation of RhoA signalling (Hernandez-Hernandez et al., 2013; May-Simera et al., 2010). Further data postulate a regulation of filopodia and lamellipodia (Hernandez-Hernandez et al., 2013; Tobin et al., 2008).

An even more direct connection between BBS and actin regulators was shown with BBS6, that directly interacts with the microtubule and actin crosslinking factor 1 (MACF1) (May-Simera et al., 2016, 2009). MACF1 connects microtubules to actin filaments to enable formation of cell-cell-contacts and is thus involved in embryonic development, cell migration and proliferation (Cusseddu et al., 2021). Via interaction with BBS6, MACF1 is required for ciliogenesis since it promotes the transport and docking of ciliary vesicles to the mother centriole (May-Simera et al., 2016). It further maintains the anchoring of microtubules at the basal body, thus enabling transport of ciliary proteins to the cilium. On the other hand, MACF1

positively regulates the phosphorylation of GSK3β, which is why it might be involved in the downstream Wnt response as well (Lin et al., 2019). These data already indicate an alternative mechanism especially for BBS proteins in affecting downstream actin structures. However, the underlying mechanisms between cilia and actin are still not fully understood.

3.3.1. The ciliary protein Bbs6 regulates filopodia length

To investigate the interplay between BBS proteins and actin in more detail, the first aim of **Manuscript I** was to identify a possible filopodia phenotype upon loss of *Bbs* proteins. Filopodia are actin-based microspikes that are formed within lamellipodia to seek out and sense the surrounding environment of the cell and are thus part of efficient cell migration (Amarachintha et al., 2015). Loss of the chaperonin-like protein Bbs6 in MEFs reduced filopodia length significantly, indicating a defect in cell sensing. Interestingly, loss of the BBSome component *Bbs8* did not result in a filopodia phenotype, although previous data indicated bbs8 being involved in the development of filopodia in zebrafish (Tobin et al., 2008). However, a detailed analysis as performed in **Manuscript I** was lacking beforehand. Bbs6 and Bbs8 were both found to affect actin structures via regulation of RhoA signalling (Hernandez-Hernandez et al., 2013), however their effect on filopodia seems to be contrary, indicating an alternative function of Bbs6 in filopodia regulations.

Bbs6 was now identified to interact with the filopodia regulator Fascin-1, suggesting a more direct regulation of Bbs6 on filopodia (**Manuscript I**). Fascin-1 is the canonical filopodia regulator since it parallelises and bundles polarised actin filaments, enabling transport of actin-based motor proteins (Pfisterer et al., 2020; Scholz et al., 2020). The structure of Fascin-1 consists of four β-trefoil domains containing two actin-binding domains N- and C-terminal within its phosphorylation sites S39 and S274 (Fig. 6 A; Hashimoto et al., 2007; Zanet et al., 2012). Since the expression level of Fascin-1 remained unchanged in *Bbs6* depleted cells, it is plausible that loss of *Bbs6* affects the actin binding capacities of Fascin-1. In support of this, BBS6 is a known assistant of CCT chaperonins. Subunits of the CCT complex localise to actin-rich structures in the cell consequently affecting actin polymerisation, and CCT chaperonins were previously found to maintain the folding of cytoskeletal proteins such as actin (Brackley and Grantham, 2010; Dunn et al., 2001; Svanström and Grantham, 2016). Thus, these data provide a basis for suggesting that BBS6 is required for the CCT-dependent folding of actin

regulators such as Fascin-1. Taken together, these data show that BBS6 might regulate filopodia independently of the downstream PCP response via direct interaction with Fascin-1.

3.3.2. Regulation of Fascin-1 in ciliogenesis

On the other hand, actin proteins are required for primary cilia development as well. Especially during early ciliogenesis, a stable actin network is needed to enable transport of preciliary vesicles to the basal body and for centrosome positioning and docking at the membrane (Dawe et al., 2009; Hong et al., 2015; Pan et al., 2007; Wu et al., 2018). The basal body is further referred to as an actin-organising centre since actin filaments are nucleated here and connected to focal adhesion complexes and the apical actin network (Antoniades et al., 2014; Farina et al., 2016; Pan et al., 2007).

As part of this thesis, a ciliogenesis phenotype was identified in *Fascin-1* depleted cells, with reduced cilia numbers, although the length of primary cilia was not altered (**Manuscript I**). These data suggest that Fascin-1 is involved in early ciliogenesis potentially by providing a stable actin network upon which ciliary vesicles are transported (Hong et al., 2015; May-Simera et al., 2016; Wu et al., 2018). Supporting these data, Fascin-1 was further found to interact with the actin scaffold Nesprin-2 (Fan et al., 2020; Jayo et al., 2016), which activates the RhoA-dependent actin network at the basal body during centrosome positioning (Dawe et al., 2009; Pan et al., 2007). Thus, it is feasible that without Fascin-1, BBS6 cannot be transported to the basal body where it is needed to maintain its chaperonin-like function and to assist Inversin in promoting PCP signalling, which is why ciliogenesis is not induced in the absence of Fascin-1. However, it is still not clear if Fascin-1 is required for BBS6 function or vice versa.

Fascin-1 was further found to localise to primary cilia independently of its capacity to bind to microtubules (**Manuscript I**), suggesting that its localisation is possibly required to structure the axonemal actin filaments (Kiesel et al., 2020). Additional data analysing the localisation of both BBS6 and Fascin-1 in Fig. 6 (below) revealed that the proteins do not completely colocalise at primary cilia, since BBS6 is predominantly found at the basal body while Fascin-1 seems to be recruited into the axoneme (Fig. 6 B, C). The function of F-actin and actin regulators inside primary cilia is mainly thought to include ectocytosis of ciliary vesicles as a way to disassemble primary cilia (Corral-serrano et al., 2020; Nager et al., 2017; Phua et al., 2017; Spencer et al., 2019; Wang et al., 2019). However, the present data showing that loss of *Fascin-1* affects cilia numbers and not cilia length does not indicate a disassembly phenotype

but rather failed induction of ciliogenesis as discussed above. Since many actin regulators were shown to be involved in early ciliogenesis (section 1.1.2. and **Publication III**), it is conceivable that Fascin-1 is rather involved in preciliary vesicle targeting instead of ciliary disassembly. In support of this, it was suggested that axonemal F-actin is required to stabilise primary cilia (Kiesel et al., 2020); thus a potential explanation for Fascin-1 inside cilia might be that it stabilises axonemal F-actin independently of its function in early ciliogenesis.

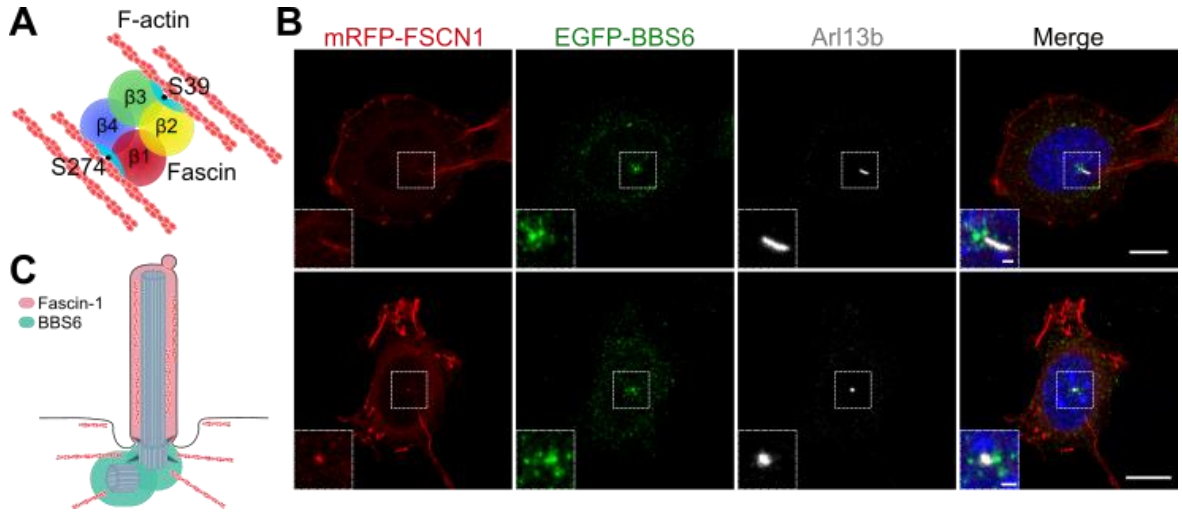

Fig. 6: Fascin-1 and BBS6 do not completely colocalise at primary cilia.

A Fascin-1 consists of four β-trefoil domains containing the two actin-binding sites located at phosphorylation sites S39 and S274. Adapted from Villari et al., 2015. **B** Overexpression of mRFP-tagged Fascin-1 in ciliated MEFs revealed a distinct localisation inside the ciliary axoneme (upper row) which is concomitant with the data from Manuscript I. EGFP-tagged BBS6 is predominantly found at and around the basal body (upper and lower row). Overexpression of both proteins for 48 hours prior fixation with 4% PFA, serum-starvation for 24 hours. Co-staining with Arl13b as a marker for the ciliary membrane. Scale bars: 10µm, magnified images: 1µm. **C** Schematic representation of the ciliary localisation of Fascin-1 inside the axoneme and BBS6 at/around the basal body. F-actin filaments were previously identified both at the basal body and inside the axoneme.

3.3.3. Fascin-1 regulates cilia-related PCP signalling

Fascin-1 was also found to interact with Inversin, suggesting that it also exerts a regulatory function in PCP signalling (**Manuscript I**). As discussed in **Manuscript I**, previous data show a potential regulation of Fascin-1 in Wnt signalling; however, the available data mostly concentrate on cancer cell lines, where the expression of Fascin-1 and Wnt signalling is severely altered (Jayo and Parsons, 2010; Shang et al., 2017). In the present study in MEFs, a non-cancerous cell model, loss of *Fascin-1* increased nuclear Cyclin D1 as well as its mRNA

expression, indicating an upregulation of canonical Wnt signalling (**Manuscript I**). It was previously shown that nuclear Cyclin D1 is associated with enhanced canonical Wnt signalling in *Bbs6* depleted kidney medullary cells (Volz et al., 2021), a result that could be recapitulated in this study. Concomitantly, loss of *Inversin* was previously shown to significantly enhance the transcription of *Cyclin D1* (Veland et al., 2013). However, it has to be noted that Cyclin D1 is also a prominent cell cycle protein and could be regulated by different pathways that lead to changes in cell cycle and subsequent regulation of Cyclin D1 (Hirayama et al., 2020; O'Connor et al., 2021). In melanoblasts, Fascin-1 was shown to regulate cell cycle progression and proliferation concomitant with changes in Cyclin D1 expression (Ma et al., 2013). Thus, it might be conceivable that changes in cell cycle rather than Wnt signalling lead to an upregulation of nuclear Cyclin D1. To exclude this fact, acetylated β-catenin was analysed as another Wnt signalling target.

As discussed in section 3.2., the acetylation of β-catenin is regulated in a cell-type and promoter specific fashion. In hTERT-RPE1 cells, acetylation of β-catenin is concomitant with activation of PCP signalling (**Publication II**). However, it seems that in MEFs, acetylation is associated with enhanced canonical Wnt signalling since loss of *Bbs6*, a well-characterised PCP protein as discussed before, increased nuclear levels of acetylated β-catenin significantly (**Manuscript I**). Loss of *Fascin-1* also enhanced nuclear levels of acetylated β-catenin, supporting the suggestion that Fascin-1 is involved in the regulation of PCP signalling.

3.3.4. Downstream regulation of PCP signalling on actin networks

PCP signalling targets many downstream processes including actin and actin-related networks which regulate cell proliferation, division and migration. Especially the regulation of Inversin in cortical actin rearrangements was previously shown and needs to be discussed to explain the downstream effect of PCP signalling (Veland et al., 2013; Werner et al., 2013). In a detailed analysis of the actin phenotype in *Inversin* depleted MEFs, a disorganisation of focal adhesion proteins concomitant with inhibited cell migration was previously observed (Veland et al., 2013). Targeting of the Rho GTPases Rac1 and RhoA, essential regulators of cell motility, to the leading edges was found to be defective, accompanied by a significant downregulation in the expression of ARP2/3 and WAVE complexes which are required for actin polymerisation. The regulation of Rho GTPases by Inversin was further shown to influence the protein level and localisation of ezrin/radixin/moesin (ERM) and the Na^+/H^+ exchanger 1 (NHE1) at the

leading edges (Veland et al., 2013). In a separate study, loss of *Inversin* results in extensive filopodia formation during mitosis and disturbed localisation of focal adhesion proteins such as paxillin (Werner et al., 2013). These changes in the subapical actin networks could be ascribed to changes in PCP signalling shown by transcriptional upregulation of canonical Wnt targets and downregulation of PCP genes such as *Gsk3β*, *Apc* and *Dvl* in the absence of *Inversin*. In testis cells, Inversin is localised to tight junctions where it regulates tight junction proteins such as ZO-1, N-cadherin and β-catenin (Li et al., 2022). Loss of *Inversin* in testis cells also affected several actin regulators such as Arp3, vimentin and the capping protein Epidermal growth factor receptor kinase substrate 8 (Eps8), concomitant with the earlier studies of Veland and colleagues. Taken together, there is a lot of overlap in the regulation of actin networks between Inversin and Bbs6 such as RhoA signalling, which can be ascribed to their cooperation in PCP signalling. Since both proteins further interact with the canonical filopodia regulator Fascin-1, additional analysis (not included in **Manuscript I**) was performed to investigate the overlap in a filopodia phenotype upon loss of *Inversin*.

Analysis of filopodia length in *Inversin* depleted MEFs via live cell imaging revealed that loss of *Inversin* significantly increased filopodia length (Fig. 7 A, B). Visualisation of Fascin-1 in fixed MEFs showed that filopodia structures in *Inversin* depleted cells were completely disrupted (Fig. 7 C), an observation that was not detected in living cells. Since filopodia are highly dynamic structures prone to collapse upon fixation, these data suggest that Inversin promotes the stability of filopodia.

Since these data are contrary to *Bbs6* depleted MEFs, where filopodia length is reduced, they suggest that Bbs6 and Inversin do not necessarily cooperate in regulating filopodia via Fascin-1 and that the downstream regulations are more complex. Further analysis is needed to investigate alternative regulatory mechanisms that explain the differences in filopodia phenotype between Bbs6 and Inversin in relation to Fascin-1.

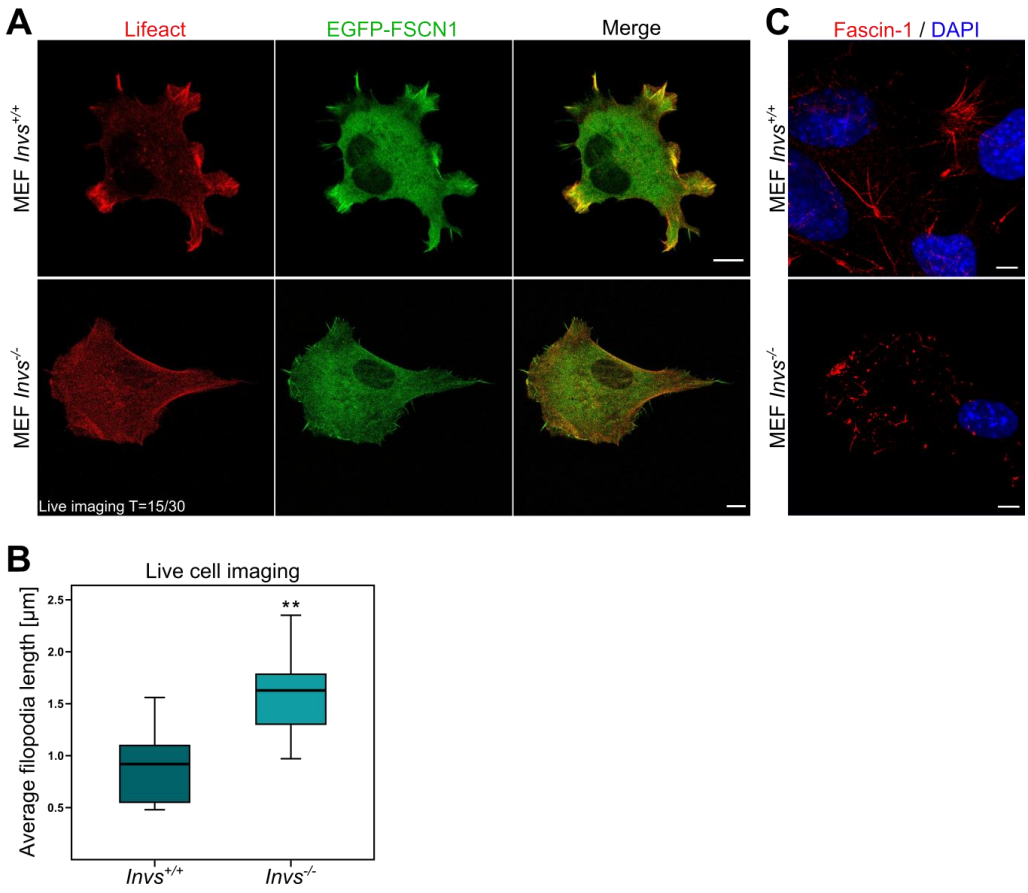

Fig. 7: Loss of *Inversin* affects filopodia.

A MEFs were cotransfected with Lifeact as a marker for the actin cytoskeleton (red) and Fascin-1 as a canonical filopodia marker (green). 48 hours after transfection, cells were imaged every five seconds for 30 timepoints on a Nikon Laser Scanning Confocal microscope and the resulting videos were analysed via FiloQuant (Jacquemet et al., 2019) to determine filopodia length. One timepoint shown here as representative image. **B** Average filopodia length of wildtype ($Inv^{+/+}$) and Inversin depleted ($Inv^{-/-}$) MEFs determined via FiloQuant shows significantly longer filopodia upon loss of Inversin. Mann-Whitney-U test: $p \leq 0.01$. Experiments were repeated three independent times. $N^{Inv+/+}=30$, $N^{Inv-/-}=21$. **C** Endogenous localisation of Fascin-1 in wildtype and *Inversin* depleted cells shows disrupted filopodia structures upon loss of *Inversin*. A detailed description of the methods can be found in Manuscript I.

Taken together, it could be shown that Bbs6 and Inversin regulate filopodia potentially via interaction with the filopodia regulator Fascin-1, however the molecular mechanisms underlying this phenotype are not completely solved. On the other hand, Fascin-1 localises to primary cilia, potentially stabilising axonemal actin. Loss of *Fascin-1* is accompanied by a ciliary phenotype, that might be resulting from defective targeting of preciliary vesicles during early ciliogenesis (Fig. 8). Loss of primary cilia subsequently leads to activation of canonical

Wnt signalling. Wnt signalling affects the downstream actin network, which might also regulate ciliogenesis again as a complex feedback mechanism. These data indicate a potential two-way mechanism for Fascin-1 in regulating actin dynamics at filopodia and primary cilia, but show that the interconnecting pathways between cilia and actin are highly complex and are still not fully understood.

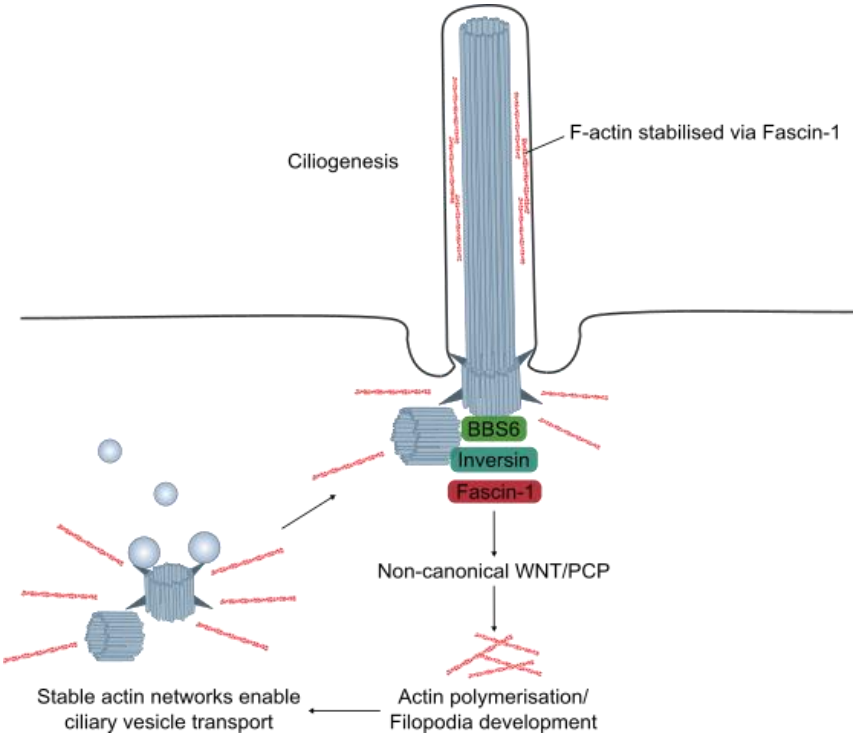

Fig. 8: Possible mechanisms for Fascin-1 in ciliary regulations.
Fascin-1 promotes actin-based development of primary cilia and interacts with BBS6 and Inversin in the regulation of downstream PCP signalling, resulting in changes of filopodia. Changes of the downstream actin network further influence actin-dependent transport of ciliary vesicles to the basal body, affecting ciliogenesis again.

3.3.5. Conclusion and outlook

Since Wnt signalling targets the downstream actin networks, **Manuscript I** aimed to clarify the interplay between Bbs proteins and the actin phenotype. The acquired data will help to understand the downstream regulation of primary cilia on cell homeostasis, thus expanding the knowledge on the molecular background of ciliopathies. Although the existing data on actin phenotypes in ciliary mutant models were expanded by showing specific defects in filopodia length upon loss of *Bbs6*, the underlying pathways are still not fully understood. The filopodia phenotypes between *Bbs6*, *Bbs8* and *Inversin* depleted cells are not concordant, although all

proteins were found to regulate PCP signalling cooperatively and Bbs6 and Inversin interact with the filopodia regulator Fascin-1. It is still unknown where the interaction between the proteins takes place: at the cilium or at filopodia, or at both places. Additional data in Fig. 6 of this thesis suggest that BBS6 and Fascin-1 might not interact at the cilium since they do not colocalise. However, a localisation of overexpressed BBS6 at filopodia was not observed and lack of robust endogenous antibodies complicates a visualisation of BBS6 at subciliary locations. Since BBS6 is required for ciliogenesis due to its chaperonin-like function, further studies should investigate if the basal body localisation of BBS6 is changed upon loss of *Fascin-1*. These data would help to understand if Fascin-1 is needed to facilitate the transport of BBS6 to the basal body as speculated in Fig. 8.

On the other hand, CCT chaperonins are known folding proteins for cytoskeletal proteins such as actin, raising the possibility that BBS6 facilitates the folding of Fascin-1 via CCT chaperonins (Brackley and Grantham, 2010; Dunn et al., 2001; Svanström and Grantham, 2016). To clarify if Bbs6 is required for folding of Fascin-1 in cooperation with CCT chaperonins, the interaction between BBS10, BBS12, the CCT complex and Fascin-1 will need further investigation.

BBS6 was previously identified to be transported into the nucleus where it affects chromatin remodelling proteins (Scott et al., 2017). Since Fascin-1 was found to interact with Nesprin-2, part of the nuclear lamina connecting the actin cytoskeleton and nuclear membrane, in regulating nuclear shape and movement (Jayo et al., 2016), it is plausible that BBS6 and Fascin-1 might interact at the nuclear envelope. Interestingly, the nuclear lamina proteins Nesprin-2 and lamin A/C were also found to impact ciliogenesis via regulation of actin networks, and Nesprin-2 interacts with the ciliopathy protein Meckelin (Dawe et al., 2009; Fan et al., 2020). In this regard, it would be of further interest to analyse potential interactions between BBS6 and Nesprin-2 or other regulators at the nuclear lamina to expand the understanding on this possible alternative nuclear function of BBS6.

Furthermore, BBS6 was previously identified to interact with MACF1 (May-Simera et al., 2016, 2009), which, like Fascin-1, is capable in regulating both microtubules and actin filaments and which was found to impact filopodia formation as well (Sanchez-Soriano et al., 2009). Since MACF1 was shown to regulate early ciliogenesis via anchoring of microtubules to the mother centriole, the role of Fascin-1 during early ciliogenesis should be investigated. Furthermore, the possible regulation of other microtubule and actin regulators in relation to

BBS could be analysed, such as the formin FHDC1 which was found to localise to the basal body where it interacts with subdistal appendage proteins (Copeland et al., 2018).

Last, the function of Fascin-1 during Wnt signalling needs further evaluation to gain a better understanding of the complex interplay between ciliogenesis, Wnt and actin networks. Besides Cyclin D1 and acetylated β-catenin, more targets need to be analysed in terms of their localisation and protein expression to better understand how exactly Fascin-1 impacts Wnt in non-cancerous cells.

Taken together, **Manuscript I** provides a valuable insight into how ciliary proteins interact with actin regulators and how these interconnections affect ciliogenesis on one side and actin structures on the other.

4. Final conclusion and remarks

In this thesis, the ciliary and non-ciliary functions of BBS proteins were investigated in more detail to provide a better understanding of the molecular mechanisms underlying the development of human ciliopathies such as Bardet-Biedl syndrome. First, the expression of *Bbs* genes in different mouse tissues was investigated to identify potential tissue-dependent functions of these proteins. In **Publication II**, the functions of respective BBS proteins were investigated in relation to ciliary disassembly and Wnt signalling during RPE development. Lastly, the downstream function of BBS proteins on actin networks was analysed which provided further data on the ciliary function of actin regulators. As discussed above, these data shed light on the ciliary and non-ciliary functions and underlying pathways regulated by BBS proteins.

In conclusion, the acquired data expands our knowledge of the molecular pathways underlying the development of human ciliopathies. They further emphasise the importance of actin and actin-related proteins in ciliogenesis that should be considered in future studies. These data provide insights that can potentially be applied to the development of therapeutics targeting these pathways. Since BBS as the flagship ciliopathy resembles many features of other ciliopathies, the data of this thesis will help to understand other cilia-related diseases as well.

5. Summary

Primary cilia are microtubule-based cell organelles that are required for the communication between cells in a tissue and the regulation of intracellular signalling pathways. They are important for tissue development and homeostasis, which is why defects in primary cilia are often associated with genetic disorders, collectively termed ciliopathies. Bardet-Biedl syndrome (BBS) as a flagship ciliopathy combines many clinical features such as retinopathies, kidney disease, obesity and polydactyly. Responsible for the occurrence of BBS are mutations in *BBS* genes, encoding proteins that are required for primary cilia development, maintenance and function. Although the ciliary function of BBS proteins has been largely discussed, recent research also suggests non-ciliary functions of BBS proteins, indicating more complex mechanisms being involved in the development of human ciliopathies. The knowledge of these complex processes is inevitable to understand ciliopathies in a broader context, enabling a better diagnosis and the potential for development of therapeutics that target these pathways.

In the current thesis, the ciliary and non-ciliary functions of BBS proteins were investigated in more detail, demonstrating complex tissue-dependent mechanisms that shed light on the signalling networks of BBS proteins on a cellular level. **Publication I** suggested that the function of BBS proteins could possibly be tissue-dependent, indicating previously unidentified regulations in specific tissues that need to be examined in more detail. While analysing the cilia-related function of BBS proteins more closely in **Publication II**, the BBS proteins BBS6 and BBS8 were found to cooperate with the Wnt signalling protein Inversin in regulating Wnt signalling and ciliary disassembly pathways. These data shed light on how BBS proteins regulate ciliogenesis in addition to their classical defined ciliary function and elucidate their role in Wnt signalling. Since Wnt signalling affects the downstream actin network, the implication on important actin-based structures such as filopodia was investigated in more detail in **Manuscript I**. These data showed that BBS6 affects filopodia via interaction with the actin regulator Fascin-1. Contrary, Fascin-1 localises to primary cilia and its loss provoked a ciliary phenotype, indicating a feedback regulation of Fascin-1 and actin in ciliogenesis. The ciliary phenotype further led to alterations of Wnt signalling, enlightening how actin proteins affect ciliogenesis and cilia-related signalling.

In summary, this thesis demonstrates how ciliary BBS proteins affect ciliogenesis and cilia-related Wnt signalling potentially in a tissue-dependent manner and provide a better understanding in how cilia, Wnt and actin regulators affect each other in complex feedback

mechanisms. These data provide a basis for studying cilia-related and unrelated functions of BBS proteins in the context of different tissues. They further emphasise the tight connection between ciliogenesis and actin proteins that should be considered in future studies to understand the complex molecular background of human ciliopathies.

6. German summary

Primärzilien sind Mikrotubuli-basierte Zellorganelle, die für die interzelluläre Kommunikation in Geweben und die Regulation von intrazellulären Signalwegen wichtig sind. Daher sind Primärzilien insbesondere für die Entwicklung und Homöostase von Organen bedeutsam, weshalb Defekte in ihrem Aufbau und ihrer Funktion oftmals mit genetischen Erkrankungen, den Ziliopathien, einhergehen. Das Bardet-Biedl-Syndrom (BBS) vereinigt viele der symptomalen Erscheinungen von Ziliopathien, wie beispielsweise Retinopathien, Nierenerkrankungen, Adipositas und Polydaktylie, weshalb die Forschung an BBS auch Rückschlüsse auf andere Ziliopathien zulässt. Verantwortlich für das Auftreten von BBS sind Mutationen in den *BBS*-Genen, welche Proteine kodieren, die für Aufbau, Instandhaltung und Funktion des Primärziliums verantwortlich sind. Die ziliären Funktionen von BBS-Proteinen sind seit langem Bestandteil der Forschung, jedoch weisen neuere Daten auch auf alternative Funktionen in der Zelle hin, die möglicherweise nicht mit dem Zilium in Verbindung stehen. Das genaue Verständnis der ziliären und nicht-ziliären Funktionen von BBS-Proteinen ist daher unabdingbar, um das Gesamtbild von Ziliopathien besser verstehen zu können, die Diagnose zu erleichtern und Behandlungsmöglichkeiten zu entwickeln.

In der vorliegenden Arbeit wurden die ziliären und nicht-ziliären Funktionen von BBS-Proteinen genauer beleuchtet, wobei neue komplexe Mechanismen und Signalwege identifiziert wurden, in denen BBS-Proteine involviert sind. **Publikation I** konzentrierte sich dabei auf die Untersuchung von möglichen Gewebe-abhängigen Mechanismen, was auf potenzielle, bislang nicht identifizierte Regulationen von BBS-Proteinen in unterschiedlichen Organen hinweist. In **Publikation II** wurde die Zilien-abhängige Funktion der BBS-Proteine näher untersucht, wobei die Regulation der BBS-Proteine BBS6 und BBS8 im Wnt-Signalweg im Zusammenhang mit ihrer Interaktion mit dem Wnt-Regulator Inversin identifiziert wurde. Diese Daten erläutern, wie BBS-Proteine die Ziliogenese alternativ zu ihrer bisher bekannten ziliären Funktion regeln

können und schlüsseln ihre Rolle im Wnt-Signalweg genauer auf. Da der Wnt-Signalweg nachgeschaltete Aktin-Netzwerke reguliert, wurde in **Manuskript I** die Auswirkung der BBS-Proteine auf Aktin-basierte Zellstrukturen wie Filopodien näher untersucht. Dabei wurde gezeigt, dass BBS6 Filopodien mittels Interaktion mit dem Aktin-Regulator Fascin-1 reguliert. Fascin-1 lokalisiert an Primärzilien und seine Abwesenheit bedingt einen ziliären Phänotypen, was ebenfalls den nachgeschalteten Wnt-Signalweg verändert. Diese Daten lassen auf Feedback-Mechanismen schließen, mit denen Fascin-1 und Aktin die Ziliogenese und ziliäre Signalwege steuern können.

Zusammenfassend konnte im Rahmen der vorliegenden Thesis gezeigt werden, wie BBS-Proteine die Ziliogenese und den ziliären Wnt-Signalweg in Gewebe-abhängigen Mechanismen steuern. Diese Arbeit bedingt dabei ein besseres Verständnis der komplexen Mechanismen, in denen Primärzilien, der Wnt-Signalweg und Aktin-Regulatoren sich gegenseitig beeinflussen. Dabei schafft diese Arbeit die Basis für weitere Studien, die die genaue Funktion der BBS-Proteine insbesondere in unterschiedlichen Organen näher beleuchtet. Weiterhin wird die enge Beziehung zwischen Aktin-Regulatoren und dem Primärzilium herausgestellt, welche in zukünftigen Studien berücksichtigt werden sollte, um die komplexen Hintergründe in der Entstehung von Ziliopathien besser zu verstehen.

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8. Supplements

8.1. Contribution to Publications

This thesis is composed of three publications and one manuscript. In the following section, my contribution to each publication will be explained in more detail.

Publication I consists of the analysis of gene expression of *Bbs* genes in different mouse tissues. For this publication, I isolated the RNA out of mouse tissues and generated cDNA. Furthermore, I helped in doing RT-qPCR of selected *Bbs* genes that was mainly performed by S. P..

In **Publication II**, the function of BBS proteins during Wnt signalling and ciliary disassembly is described. My part included performing the interaction studies (GFP traps) between the BBS proteins BBS2/BBS6 and the Wnt regulator Inversin. Furthermore, I analysed the ciliary localisation of Inversin in wildtype and *Bbs6* depleted mouse kidney medullary cells and did the quantification and analysis of the resulting immunofluorescence pictures. I also helped S. P. with western blots analysing the protein HEF1 in *Bbs8* depleted and MG132 treated cells.

Manuscript I describes the role of BBS proteins in filopodia regulations via interaction with Fascin-1 and the potential ciliary function of Fascin-1. Most of these data were acquired by me or S. B., who worked under my supervision. I have also written the complete manuscript and performed literature research.
For analysing filopodia length, I performed live cell imaging of *Bbs6*, *Bbs8* and *Inversin* depleted MEFs. I analysed the resulting videos of *Bbs6* and *Inversin* depleted cells, whereas S. B. did the analysis of *Bbs8* MEFs. I performed immunofluorescence stainings of endogenous and overexpressed Fascin-1 in MEFs and investigated the interaction between Fascin-1 and Inversin and BBS6/BBS8 via GFP traps. I isolated protein out of *Bbs6* depleted MEFs and S. B. and I performed western blots of different proteins. The analysis of Bbs6 in ubiquitination of Fascin-1 including co-immunoprecipitations was also performed by me. I performed siRNA transfections for *Fascin-1* in MEFs and S. B. and I were both involved in the subsequent RNA isolation, cDNA synthesis and RT-qPCR analysis. Under my supervision, S. B. performed knockdown of *Fascin-1* in wildtype and *Bbs6* depleted MEFs and analysed cilia numbers and

length. S. B. further validated the knockdown of *Fascin-1* via immunofluorescence and investigated the ciliary localisation of Fascin-1 mutations.

Publication III is a literature review on the interplay between cilia and actin. I did the literature research and writing of this manuscript while V. K. created the figures. I further provided an immunofluorescence image showing the actin cytoskeleton and cilia markers for figure 1.

8.2. Abbreviations

ALMS	Alström syndrome
APC	Adenomatous polyposis coli
ARF	ADP-ribosylation factor
ARL6	ADP-ribosylation factor-like protein 6
BB	Basal body
BBIP1	BBSome-interacting protein 1
BBS	Bardet-Biedl syndrome
BCL9	B-cell lymphoma 9
C8orf37	Chromosome 8 open reading frame 37 protein
CBP	CREB-binding protein
CCT	Chaperonin containing TCP-1
CEP	Centrosomal protein
CK1	Casein kinase 1
Cobl	Cordon-Bleu WH2 Repeat Protein
CP	Ciliary pocket
DA	Distal appendage
DAAM1	Dishevelled-associated activator of morphogenesis 1
DNA	Desoxyribonucleic acid
DVL	Dishevelled
ER	Endoplasmic reticulum
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FZD	Frizzled

GSK3β	Glycogensynthase kinase 3β
GTP	Guanosine triphosphate
JNK	c-Jun N-terminal kinase
JUN	c-Jun
IFT	Intraflagellar transport
JATD	Jeune syndrome
JBTS	Joubert syndrome
LCA	Leber Congenital Amaurosis
LEF	Lymphoid enhancer-binding factor
LRP	Low-density lipoprotein receptor-related protein
LZTFL1	Leucine zipper transcription factor-like protein 1
MACF1	Microtubule and actin crosslinking factor 1
mTOR	mammalian Target of rapamycin
MKKS	McKusick–Kaufman syndrome
MKS	Meckel syndrome
NPHP	Nephrocystins/Nephronophthisis
OFD1	Oro-facial-digital syndrome type 1
P	Phosphorylation
PCP	Planar cell polarity
PDGF	Platelet-derived growth factor
PM	Plasma membrane
Pygo	Pygopus
RHOA	Ras homolog family member A
ROCK	Rho-associated, coiled-coil-containing protein kinase

SCF	Skp1-Cullin1-F-box
SDA	Subdistal appendage
SDCCAG8	Serologically defined colon cancer antigen 8
Shh	Sonic hedgehog
SLS	Senior-Loken syndrome
TCF	T-cell factor
TGFβ	Transforming growth factor β
TriC	T-complex protein-1 ring complex
TRIM32	Tripartite motif containing 32
TTC8	Tetratricopeptide repeat domain protein 8
Wash	Wiskott–Aldrich syndrome protein and SCAR homologue
WDPCP	WD repeat containing planar cell polarity effector protein

8.3. Index of Figures

8.4. Index of Tables

8.5. Acknowledgements

8.6. Curriculum vitae

8.7. Declaration of honour

I declare that I have written the present thesis on my own while using only the given methods.

I have not handed in this thesis at another faculty or university before and I have not attempted to reach the academic degree Dr. rer. nat. before.

I certify that all information contained in this application is correct to the best of my knowledge.

Mainz,

_____

Lena Brücker