Rpgrip1l controls ciliary gating by ensuring the proper amount of Cep290 at the vertebrate transition zone

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INTRODUCTION

The spatiotemporal regulation of cellular processes such as proliferation, apoptosis, migration, specification, and differentiation depends on the cell's ability to transduce signals from the environment into the cell's interior. In nearly all mammalian cells, the primary cilium is dedicated to signal reception and transduction. Consequently, dysfunctional primary cilium result in severe often deadly human diseases, collectively called ciliopathies (Reiter and Leroux, 2017). The current treatment of ciliopathies is restricted to symptomatic therapies and a curative medication against ciliopathies is missing (McIntyre et al., 2013). In many cases, ciliopathies are caused by mutations in genes encoding transition zone (TZ) proteins (Hildebrandt et al., 2011; Czarnecki and Shah, 2012). As the TZ functions as the ciliary gatekeeper governing ciliary protein import and export (Betleja and Cole, 2010; Craige et al., 2010; Omran, 2010; Benzing and Schermer, 2011; Czarnecki and Shah, 2012; Garcia-Gonzalo and Reiter, 2012; Reiter et al., 2012; Garcia-Gonzalo and Reiter, 2017; Jensen and Leroux, 2017), a defective TZ can affect the proper formation of cilia and alter the transduction of signaling pathways (Reiter and Skarnes, 2006; Vierkotten et al., 2007; Chih et al., 2011; Garcia-Gonzalo et al., 2011; Williams et al., 2011; Warburton-Pitt et al., 2012; Jensen et al., 2015; Yee et al., 2015; Li et al., 2016; Shi et al., 2017; Weng et al., 2018; Lewis et al., 2019). Thus, the investigation of the molecular mechanisms underlying TZ function is an important prerequisite for the development of curative ciliopathy therapies.

In this study, we shed light on the role of Rpgrip11 in regulating the ciliary gating function of the TZ. Our previous investigations revealed that mutations in RPGIRIP1L cause deadly ciliopathies (Delous et al., 2007), that Rpgrip11 localizes to the vertebrate TZ (Gerhardt et al., 2015), and that it is a decisive factor in vertebrate TZ...
Rpgrip1l and Cep290 but not nephrocystin (Nphp1), Nphp4, and Inversin (Invs) function as ciliary gatekeepers in mouse embryonic fibroblasts (MEFs).

Among the proteins allowed to cross the TZ are receptors and mediators of signaling pathways essential for proper development. Examples for such proteins are ADP ribosylation factor-like GTPase 13B (Arl13b), somatostatin receptor 3 (Sstr3), smoothened (Smo), and spindle pole-associated protein 1 (Cspp1) was an interesting object of investigation because its truncation in humans results in an important task to unveil possible relationships between these gatekeeper proteins in order to understand the mechanisms that govern ciliary protein composition. In this context, centrosome and spindle pole-associated protein 1 (Cspp1) was an interesting object of investigation because its truncation in humans results in ciliopathies caused by mutations in Rpgrip1l.

RESULTS

However, we could not detect an alteration of the ciliary Smo amount in Rpgrip1l−/− MEFs (Gerhardt et al., 2015), raising the question whether the effect of Rpgrip1l is Arl13b-specific or whether it functions as a more general ciliary gatekeeper at the vertebrate TZ. To answer this question, we analyzed the ciliary Sstr3 amount. It has been known for a long time that Sstr3 localizes to cilia of neuronal cells (Händel et al., 1999). More recently, it was shown that Sstr3 is also present in cilia of retinal pigment epithelial (RPE-1) cells (Klinger et al., 2014). We were now able to visualize endogenous Sstr3 in cilia of MEFs. Importantly, the amount of Sstr3 was decreased in Rpgrip1l−/− MEFs (Figure 1A) demonstrating that Rpgrip1l exerts a TZ gatekeeper function.

We next aimed to investigate how Rpgrip1l implements this function. Several proteins function as ciliary gatekeepers and it is an important task to unveil possible relationships between these gatekeeper proteins in order to understand the mechanisms that govern ciliary protein composition. In this context, centrosome and spindle pole-associated protein 1 (Cspp1) was an interesting object of investigation because its truncation in humans results in ciliopathies caused by mutations in Rpgrip1l.

FIGURE 1: In mouse cells, ciliary gating is disturbed by the loss of Rpgrip1l but not by the loss of Nphp4. (A) Immunofluorescence on MEFs obtained from WT (n = 5) and Rpgrip1l−/− (n = 5) embryos. At least 10 cilia per embryo were used for quantifications (Σ(WT) = 62 cilia, Σ(Rpgrip1l−/−) = 72 cilia). (B) Immunofluorescence on MEFs obtained from WT (Arl13b: n = 4; Sstr3: n = 3) and Nphp4−/− (Arl13b: n = 4; Sstr3: n = 3) embryos. At least 20 cilia per embryo were used for quantifications (Arl13b: Σ(WT) = 83 cilia, Σ(Nphp4−/−) = 171 cilia; Sstr3: Σ(WT) = 134 cilia, Σ(Nphp4−/−) = 92 cilia). (A, B) The ciliary axoneme is stained in green by acetylated α-tubulin, the BB is stained in blue by γ-tubulin. The scale bars represent a length of 0.5 µm. Data are shown as mean ± SEM. Asterisks denote statistical significance according unpaired t tests with Welch’s correction (***P < 0.001) (A: t(25) = 6.54, P < 0.0001; B: Arl13b: t(46) = 0.7483, P < 0.4581; Sstr3: t(80) = 1.807, P < 0.0745).
amount of Csp1 at the ciliary base of Rpgrip1l−/− MEFs but we could not detect any alteration (Supplemental Figure S1A), indicating that Rpgrip1l exerts its ciliary gatekeeper function independently of Csp1. Recently, a potential link between Rpgrip1l and the septin proteins was discussed (Patnaik et al., 2018). Septins are known ciliary gatekeepers in vertebrates localizing at the proximal end of the axoneme in MEFs (Hu et al., 2010). We measured the ciliary amount of two different septins, Septin 2 (Sept2) and Septin 7 (Sept7), in Rpgrip1l−/− MEFs. Neither Sept2 nor Sept7 was altered by the loss of Rpgrip1l (Supplemental Figure S1, B and C). We then turned to the TZ proteins Nphp4, Nphp1, centrosomal protein 290 (Cep290) and Invns whose amount at the vertebrate TZ is decreased in the absence of Rpgrip1l (Wiegering et al., 2018a). Previous studies in the invertebrates Chlamydomonas reinhardtii and/or Caenorhabditis elegans demonstrated that ciliary gating is regulated by the TZ proteins Nphp4, Nphp1, Cep290, and Invns (Ira et al., 2010; Williams et al., 2011; Warburton-Pitt et al., 2012; Awata et al., 2014; Li et al., 2016), raising the possibility that Rpgrip1l might govern ciliary gating by controlling the amount of one of these proteins at the TZ. However, it is unknown whether these proteins function as ciliary gatekeepers in vertebrates and hence we quantified the ciliary amount of Arl13b and Sstr3 in Nphp4−/−, Nphp1−/−, Cep290−/−, and Invns−/− mouse cells. The ciliary amount of both Arl13b and Sstr3 was unaffected in Nphp4−/− MEFs (Figure 1B). To analyze TZ assembly, we had previously inactivated Nphp1, Cep290, and Invns in NIH3T3 cells (immortalized MEFs; Wiegering et al., 2018a). In the current study, we used these cells to investigate a possible involvement of these proteins in ciliary gating. To be able to perform comparative analyses in this context, we also inactivated Rpgrip1l in NIH3T3 cells (Supplemental Figure S2). As observed in MEFs (Hergardt et al., 2015), ciliary length was increased in Rpgrip1l−/− NIH3T3 cells (Supplemental Figure S2C). Moreover, the ciliary amount of Arl13b and Sstr3 was decreased in Rpgrip1l−/− NIH3T3 cells (Figure 2, A and B). In contrast to these cells, Nphp1−/− and Invns−/− NIH3T3 cells did not show an altered ciliary amount of Arl13b and Sstr3 (Figure 2, A and B) indicating that the decreased amount of Nphp1 and Invns in Rpgrip1l−/− MEFs is not the reason for the reduced ciliary Arl13b and Sstr3 amount. Importantly, Cep290 deficiency causes a decrease of the ciliary Arl13b and Sstr3 amount (Figure 2, A and B) making it conceivable that the reduced amount of Cep290 might provoke the diminished amount of Arl13b and Sstr3 in the absence of Rpgrip1l.

Restoration of the Cep290 amount at the TZ of Rpgrip1l−/−, NIH3T3 and RPGRIP1L−/− HEK293 cells rescues the ciliary Arl13b and Sstr3 amount

To test this hypothesis, we enhanced the amount of Cep290 at the TZ of Rpgrip1l−/− NIH3T3 cells by transfecting a plasmid that encodes a Flag-Rpgrip1l fusion protein. In addition, to test for the conservation of the functional relationship between these two TZ proteins in humans, we transfected a plasmid that encodes a GFP-hCep290 fusion protein into RPGRIP1L−/− HEK293 cells. In a previous study, we revealed a reduced amount of Cep290 at the TZ of Rpgrip1l−/− MEFs and RPGRIP1L−/− HEK293 cells (Wiegering et al., 2018a). In line with this, a reduction of Cep290 at the ciliary TZ of Rpgrip1l−/− NIH3T3 cells was observed in the present study (Supplemental Figure S4, A and F). In NIH3T3 and HEK293 cells, the transfected Cep290 fusion protein was located at the TZ (Supplemental Figure S4, A and H). In contrast to the Flag-Rpgrip1l fusion protein in NIH3T3 cells and the GFP-hCep290 fusion protein in HEK293 cells, we could not detect a transfected Myc-nNhph1 fusion protein at the TZ of Rpgrip1l−/− NIH3T3 or RPGRIP1L−/− HEK293 cells (Supplemental Figure S3). Most likely, Rpgrip1l functions as a TZ scaffold for Nphp1 but not for Cep290.

The transfection of the plasmid encoding the Flag-mCep290 fusion protein into Rpgrip1l−/− NIH3T3 cells as well as the transfection of the plasmid encoding the GFP-hCep290 fusion protein into RPGRIP1L−/− HEK293 cells at least partially restored the amount of Cep290 at the TZ (Supplemental Figure S4, E, F, K, and L). Importantly, the rescued amount of Cep290 restored the ciliary amount of Arl13b and Sstr3 in Rpgrip1l−/− NIH3T3 (Figure 3, A–C) and the ciliary amount of Arl13b in RPGRIP1L−/− HEK293 cells (Figure 3, D and E). HEK293 cells lack Sstr3, preventing the analysis of its localization in the absence of RPGRIP1L and its rescue by Cep290 overexpression (War and Kumar, 2012). Our results indicate that Rpgrip1l controls ciliary gating via ensuring the proper amount of Cep290 at the TZ, both in mouse and human cells.

Cilia of NIH3T3 and HEK293 cells are elongated by the loss of Rpgrip1l (Wiegering et al., 2018a) (Supplemental Figure S4, C and J). Interestingly, the increased cilia length in the absence of Rpgrip1l was not rescued by the expression of the Flag-mCep290 fusion protein in Rpgrip1l−/− NIH3T3 cells or the GFP-hCep290 fusion protein in RPGRIP1L−/− HEK293 cells (Supplemental Figure S4, C and J). Thus, contrary to its ciliary gating function, the role of Rpgrip1l in controlling ciliary length is not mediated by Cep290.

To verify the functionality of the Cep290 fusion protein, we transfected the plasmid encoding Flag-mCep290 into Cep290−/− NIH3T3 cells. The transfected Cep290 fusion protein was located at the TZ in Cep290−/− NIH3T3 cells (Supplemental Figure S4A) and the amount of Cep290 at the TZ was restored (Supplemental Figure S4, E and G). Moreover, the decreased cilia length observed in the absence of Cep290 (Wiegering et al., 2018a) was rescued by the expression of the Flag-mCep290 fusion protein (Supplemental Figure S4D), demonstrating the functionality of the transfected protein in NIH3T3 cells.

Eupatilin treatment rescues ciliary gating in Rpgrip1l-negative MEFs

A recent report showed that eupatilin rescues ciliary gating in CEP290−/− human cells by replacing the function of CEP290 in TZ recruitment of Nphp5 (NPHP5; alias IQCB1) (Kim et al., 2018). Since we showed above that Rpgrip1l function on ciliary gating was mediated by the control of Cep290 TZ amounts, we hypothesized that eupatilin would also rescue the ciliary gating defect in Rpgrip1l-deficient cells. Indeed, the treatment of Rpgrip1l−/− NIH3T3 cells with eupatilin restored the ciliary amount of both Arl13b and Sstr3 (Figure 4, A, B, and D), confirming our hypothesis. The enhanced cilia length in Rpgrip1l−/− NIH3T3 cells was not rescued by eupatilin (Figure 4I). We also verified that eupatilin treatment of Cep290−/− NIH3T3 cells restored the reduced amount of Arl13b and Sstr3 (Figure 4, A, C, and E), but not ciliary length alteration (Figure 4J), in these cells. Furthermore, we analyzed the TZ amount of Nphp5 and found that it was significantly reduced in both Rpgrip1l−/− and Cep290−/− NIH3T3 cells (Figure 4, F–H). Eupatilin treatment rescued the amount of Nphp5 completely in Rpgrip1l−/− NIH3T3 cells (Figure 4G) and more partially in Cep290−/− NIH3T3 cells (Figure 4H). Thus, we conclude that Rpgrip1l functions in ciliary gating upstream of Nphp5, via ensuring the proper amount of Cep290 at the TZ.

DISCUSSION

Primary cilia mediate numerous signaling pathways thereby ensuring proper development and homeostasis. In this context, the intracellular concentration of proteins involved in these signaling pathways is of enormous importance. Consequently, ciliary import
and export and hence ciliary protein composition has to be tightly controlled. This control is implemented by the TZ. Since mutations in genes encoding TZ proteins result in ciliopathies (Hildebrandt et al., 2011; Czarnecki and Shah, 2012; Reiter and Leroux, 2017), current cilia research aims to uncover mechanisms underlying TZ assembly and function. However, little is known about these mechanisms in vertebrates. Recently, we described Rpgrip1l as a decisive factor in vertebrate TZ assembly (Wiegering et al., 2018a). In this context, Rpgrip1l deficiency leads to a reduced amount of Cep290, Nphp1, Nphp4, and Invs at the TZ (Wiegering et al., 2018a). Rpgrip1l, Cep290, Nphp1, Nphp4, and Invs were previously shown to govern ciliary gating in C. reinhardtii and/or C. elegans (Craigie et al., 2010; Williams et al., 2011; Warburton-Pitt et al., 2012; Awata et al., 2014; Li et al., 2016; Lin et al., 2018). However, loss of Nphp1, Nphp4, and Invs did not alter the ciliary amount of Arl13b or Sstr3 in MEFs and NIH3T3 cells (Figures 1B and 2), indicating that they are not involved in gating these proteins in vertebrate primary cilia. Remarkably, several reports point to cell type-specific functions of some TZ proteins (Garcia-Gonzalo et al., 2011; Rachel et al., 2015; Lambacher et al., 2016; Wiegering et al., 2018a; Lewis et al., 2019) making a potential regulation of ciliary gating by Nphp1, Nphp4, and Invs in other vertebrate cell types conceivable. Rpgrip1l−/− and Cep290−/− mice have a much more severe phenotype than Nphp1−/−, Nphp4−/− and Invs−/− mice.

FIGURE 2: Loss of Cep290 but not Nphp1 or Invs impairs ciliary gating in mouse cells. (A, B) Immunofluorescence on NIH3T3 cells. The ciliary axoneme is stained in green by acetylated α-tubulin. The scale bars represent a length of 0.5 µm. At least 20 cilia per clone were used for quantification. Data are shown as mean ± SEM. Asterisks denote statistical significance according to one-way ANOVA and Tukey HSD tests (***P < 0.001) (A: F(7,160) = 26.09, P < 0.0001; B: F(7,147) = 133.6, P < 0.0001).
(Mochizuki et al., 1998; Morgan et al., 1998; Chang et al., 2006; Delous et al., 2007; McEwen et al., 2007; Vierkotten et al., 2007; Jiang et al., 2008; Jiang et al., 2009; Louie et al., 2010; Besse et al., 2011; Lancaster et al., 2011; Won et al., 2011; Gerhardt et al., 2013; Hynes et al., 2014; Chen et al., 2015; Laclef et al., 2015; Li et al., 2015; Rachel et al., 2015; Wiegner et al., 2018a; Andreu-Cervera et al., 2019; Choi et al., 2019). Moreover, mutations in Rpgrip1L and CEP290 result in more severe human ciliopathies than mutations in NPHP1, NPHP4, or INVS (Zaghloul and Katsanis, 2010; Szymanska and Johnson, 2012; Madhivanan and Aguilar, 2014; Mitchison and Valente, 2017). On the one hand, these differences might reflect that Rpgrip1L and CEP290 function as ciliary gatekeepers in vertebrates while Nphp1, Nphp4, and Invs do not. On the other hand, these differences might be based on the fact that Rpgrip1L and CEP290 exert additional functions in the cytoplasm, for example, the regulation of protein degradation systems (Gerhardt et al., 2015; Struchtrup et al., 2018) or the organization of the cytoplasmic microtubule network (Kim et al., 2008).

FIGURE 3: Rescue of the Cep290 amount at the TZ of Rpgrip1L+/+ NIH3T3 and Rpgrip1L−/− HEK293 cells restores the ciliary Arl13b and Sstr3 amount. (A–C) Rpgrip1L+/+ and Rpgrip1L−/− NIH3T3 cells were either untransfected or transfected with TF-Ctrl or pFlag-mCep290. (A) Immunofluorescence on Rpgrip1L+/+ and Rpgrip1L−/− (clone 10-61) NIH3T3 cells. The ciliary axoneme is stained in green by acetylated α-tubulin and Arl13b or Sstr3 is stained in red. The scale bars represent a length of 0.5 µm. (B, C) Normalized ciliary amount of Arl13b (B) and Sstr3 (C). At least 30 cilia per clone were used for quantification. Data are shown as mean ± SEM. Asterisks denote statistical significance according to one-way ANOVA and Tukey HSD tests (**P < 0.01; ***P < 0.001) (B: F(5, 400) = 146.8, P < 0.0001; C: F(5, 166) = 128.6, P < 0.0001). (D, E) Rpgrip1L+/+ and Rpgrip1L−/− HEK293 cells were either untransfected or transfected with eGFP-hCEP290 or TF-Ctrl. (D) Immunofluorescence on Rpgrip1L+/+ and Rpgrip1L−/− (clone 1–7) HEK293 cells. The ciliary axoneme is stained in green by acetylated α-tubulin and Arl13b is stained in red. The scale bars represent a length of 0.5 µm. (E) Normalized ciliary amount of Arl13b. At least 10 cilia (Rpgrip1L+/+ HEK293 cells) or 20 cilia (Rpgrip1L−/− HEK293 cells) per clone were used for quantification. Data are shown as mean ± SEM. Asterisks denote statistical significance according to one-way ANOVA and Tukey HSD tests (**P < 0.001) (F(5, 104) = 142, P < 0.0001).
FIGURE 4: Eupatilin treatment rescues ciliary gating in the absence of Rpgrip1l. (A, B) Immunofluorescence on Rpgrip1l+/+, Rpgrip1l−/− (clone 10-61) and Cep290−/− NIH3T3 cells. The ciliary axoneme is stained in green by acetylated α-tubulin. Arl13b or Sstr3 is stained in red. The scale bars represent a length of 1 µm. (B–E) Normalized ciliary amount of Arl13b (B, C) and Sstr3 (D, E). At least 20 (D, E) or 30 cilia (B, C) per clone were used for quantification. The same quantification of WT serves as comparison to Rpgri1l-negative and Cep290-negative cells, respectively (B and C; D and E). Data are shown as mean ± SEM. Asterisks denote statistical significance according to one-way ANOVA and Tukey HSD tests (***, P < 0.001) (B: F(5, 447) = 117.1, P < 0.0001; C: F(11, 690) = 163.4, P < 0.0001; D: F(5, 183) = 47.48, P < 0.0001; E: F(11, 393) = 35.27, P < 0.0001). (F) Immunofluorescence on Rpgrip1l+/+, Rpgrip1l−/− (clone 10-61) and Cep290−/− NIH3T3 cells. The ciliary axoneme is stained in green by acetylated α-tubulin. Nphp5 is stained in red. The scale bars represent a length of 1 µm. (G, H) Normalized ciliary amount of Nphp5. At least
Rpgrip1l does not function as scaffold for Cep290 and Cspp1 at the TZ

Formerly, we demonstrated that Rpgrip1l deficiency does not affect the overall cellular amount of Cep290 but its proper amount at the vertebrate TZ (Wiegiering et al., 2018a). There is a perennial debate about the function(s) of Rpgrip1l. Does it predominantly serve as a structural TZ anchor or scaffold protein and interacts with other proteins, thereby ensuring their localization and proper amount at the TZ, or does it control the TZ localization and amount of proteins by exerting additional functions, for example, regulating protein degradation systems, functioning as a TZ assembly factor, establishing a ciliary zone of exclusion that excludes signal transduction proteins, etc. (Coene et al., 2011; Williams et al., 2011; Gerhardt et al., 2015; Jensen et al., 2015; Assis et al., 2017; Shi et al., 2017; Struchtrup et al., 2018; Wiegiering et al., 2018a; Wiegiering et al., 2018b)? In this study, the Flag-mCep290 and the GFP-hCEP290 fusion proteins were able to localize to the TZ in the absence of Rpgrip1l (Supplemental Figure S4, A and H), indicating that Rpgrip1l does not function as a structural scaffold for the TZ presence of Cep290. In line with this assumption, it was not shown yet that Rpgrip1l interacts with Cep290. To stress this point, we also transfected a plasmid encoding a Myc-mNphp1 fusion protein into Rpgrip1l–/– NIH3T3 and Rpgrip1l+/– HEK293 cells (Supplemental Figure S3). It was reported before that Nphp1 interacts with Rpgrip1l (Sang et al., 2011). Since Myc-mNphp1 was not present at the TZ in the absence of Rpgrip1l (Supplemental Figure S3, B and D), we suggest that Rpgrip1l functions as a structural anchor for Nphp1 but not for Cep290. The mechanism by which Rpgrip1l regulates the TZ amount of Cep290 is thus not understood, and it will be an exciting future challenge to address this question.

For example, it would be conceivable that Rpgrip1l regulates the amount of Cep290 via interaction with centriolar satellites. It is predicted that Cep290 is part of a satellite subnetwork consisting of pericentriolar material 1 (PCM1), SSX family member 2 interacting protein (SSX2IP), orofaciocutaneous syndrome protein 1 (OFD1, centriole and centriolar satellite protein), synaptic vesicle glycoprotein 2B (SV2B; also known as KIAA0735), and CEP290 (Gupta et al., 2015), and that Rpgrip1l is a potential interaction partner of this satellite subnetwork by interacting at least with PCM1 and SSX2IP (Gupta et al., 2015). PCM1 is a major component of centriolar satellites involved in the recruitment of Ceps such as centrin and ninein as well as the organization of a cytoplasmic microtubule network (Kubo et al., 1999; Dammermann and Merdes, 2002; Kubo and Tsukita, 2003). Several studies have shown physical and functional interactions between PCM1 and CEP290 (Chang et al., 2006; Kim et al., 2008; Gupta et al., 2015). However, the loss of PCM1 decreases the localization of CEP290 at centriolar satellites but does not affect the centrosomal/basal body (BB) accumulation of CEP290 (Kim et al., 2008; Stowe et al., 2012; Odbashi et al., 2019). In contrast to that, loss of SSX2IP leads to a reduced amount of CEP290 at the TZ (Klinger et al., 2014). SSX2IP is known to be involved in microtubule anchoring, centrosome maturation, and ciliogenesis as well as being an important effector protein in the FOXJ1 regulatory network (Bärenz et al., 2013; Hori et al., 2014; Hori et al., 2015; Mukherjee et al., 2019). On ciliogenesis, SSX2IP accumulates around the BB (Klinger et al., 2014) and loss of SSX2IP decreases ciliary length in RPE-1 cells (Hori et al., 2014; Klinger et al., 2014). Taken together, Rpgrip1l could regulate the TZ amount of Cep290 by directly regulating SSX2IP, which in turn regulates Cep290.

It was shown before that the proper amount of Rpgrip1l at the TZ depends on Cspp1 and that Rpgrip1l and Cep290 are directly interacting (Patzke et al., 2010). Moreover, mutations in CSPP1 disturb ciliary protein composition (e.g., reduced ciliary Arl13b amount; Tuz et al., 2014) and cause Joubert syndrome and Meckel syndrome (Shaheen et al., 2014). Interestingly, Rpgrip1l was not required for the ciliary localization of Cspp1 (Supplemental Figure S1A). Based on these facts, we propose that Cep290 is at the top of the “ciliary gating hierarchy,” and it would be an interesting future task to monitor if gating defects in Cep290-negative cells are indeed mediated by a decreased TZ amount of Rpgrip1l.

Rpgrip1l controls ciliary gating via Cep290

Here we show that Rpgrip1l controls ciliary gating via ensuring the proper amount of Cep290 at the TZ (Figure 3). The role of Cep290 in ciliary gating is addressed by several studies in which a reduced amount of ciliary membrane proteins like Arl13b and Ac3 in the absence of Cep290 has been shown (Craigie et al., 2010; Li et al., 2016; Shimada et al., 2017; Kilander et al., 2018; Kim et al., 2018; Molinari et al., 2019). How Cep290 implements this function is not yet clearly understood, but it was shown that Cep290 governs ciliary protein composition by interacting with Nphp5 (Barbelanne et al., 2015a; Li et al., 2016; Shimada et al., 2017; Kim et al., 2018). Cep290 binds Nphp5 thereby covering the calmodulin binding of Nphp5 and promoting the recruitment of Nphp5 to the TZ (Kim et al., 2018). In the absence of Cep290, the Nphp5 amount at the TZ is reduced, and this amount is restored by eupyatin treatment, which inhibits calmodulin binding to Nphp5 (Kim et al., 2018). In our study, we observed a similar rescue of ciliary amounts of Arl13b and Str3, and of Nphp5 TZ amounts, by eupyatin treatment in Rpgrip1l-deficient cells (Figure 4). Together with the rescue of the Arl13b and Str3 amount by transfection of tagged Cep290 (Figure 3), these data underpin our assertion that Rpgrip1l exerts a gatekeeper function via Cep290 and Nphp5 (Figure 5). Interestingly, the phenotype of Nphp5-negative mice is not as striking as the phenotype of Cep290-mutant or Rpgrip11-mutant mice, raising the question whether ciliary gating can really be regulated by Nphp5. Nonetheless, it was shown that the phenotype of Nphp5-mutant mice shows similarities to the phenotype of Cep290-mutant mice and that patients with mutations in NPHP5 can develop similar ciliopathy syndromes than patients with mutations in CEP290 (Otto et al., 2005; Chang et al., 2006; Helou et al., 2007; Stone et al., 2011; Ronquillo et al., 2016). It was also shown that the interaction of Cep290 and Nphp5 is required for ciliogenesis (Barbelanne et al., 2013) and that Nphp5 as well as Cep290 regulates components of the BBSome (octameric protein complex consisting of Bardet-Biedl syndrome) (Barbelanne et al., 2015a). In addition, Nphp5 interacts with components of the exocyst complex, a protein complex involved in exocytosis and thereby ciliogenesis (Zuo et al., 2009;
mutant NIH3T3 cells feature opposite ciliary length alterations (Figures 4, I and J and Supplemental Figure S4, C and D), which probably cannot be explained by the corresponding defect in ciliary gating, different mechanisms have to be affected in the respective mutant. It has been shown that Cep290 regulates ciliary localization of BBS4 via interaction with PCM-1, thereby regulating BBSome integrity and ciliary trafficking (Stowe et al., 2012; Klinger et al., 2014; Kobayashi et al., 2014; Barbelanne et al., 2015a). In this context, Cep290 is involved in the recruitment of the small GTPase Rab8 to the cilium. Rab8 regulates vesicle trafficking and has been shown to collaborate with the BBS protein complex involved in ciliary membrane formation (Nachury et al., 2007; Yoshimura et al., 2007; Kim et al., 2008; Tsang et al., 2008). Taken together, the reduction of ciliary length in Cep290−/− NIH3T3 cells could result from a failed recruitment of BBSome components and Rab8 followed by a misregulated vesicle trafficking and disrupted ciliary membrane elongation. In this regard, the reduction of Cep290 at the TZ in Rpgrip1l−/− NIH3T3 cells by around 50% does not seem strong enough to disrupt Cep290-dependent vesicle trafficking and ciliary membrane elongation in this mutant.

Instead it has been shown that Rpgrip1l interacts with Myosin Va (Assis et al., 2017), a motor protein required for preciliary vesicle transportation to the mother centriole during ciliogenesis (Wu et al., 2018). The loss of Myosin Va leads to decreased ciliogenesis in RPE-1 and murine inner medullary collecting duct (IMCD3) cells and it is assumed that an increased Myosin Va amount at the cilial base leads to elongated cilia (Assis et al., 2017; Kohli et al., 2017; Copeland, 2020). As we discussed in a previous study (Wiegering et al., 2018b), mammalian Myosin Va is closely related to Myosin V in Drosophila melanogaster (Bonafé and Sellers, 1998). Myosin V is a substrate of proteasomal degradation (Pocha et al., 2011), making it very likely that Myosin Va is likewise degraded by the proteasome. Since Rpgrip1l regulates the proteasome specifically at the ciliary base (Gerhardt et al., 2015), Rpgrip1l could regulate the degradation of Myosin Va via the ciliary proteasome. A loss of Rpgrip1l would lead to an increased amount of Myosin Va at the ciliary base, resulting in an increased vesicle transport during ciliogenesis and an

FIGURE 5: Graphical abstract of ciliary gating defects and their rescue in Rpgrip1l- and Cep290-negative NIH3T3 cells. Rpgrip1l controls ciliary gating via ensuring the proper amount of Cep290 at the TZ, which in turn regulates the ciliary amount of Nphp5. In the absence of Rpgrip1l, the ciliary amount of Cep290 and Nphp5 is reduced. This leads to ciliary gating defects indicated by lower ciliary Arl13b and Strt3 levels. In Cep290-negative cells, the ciliary amount of Rpgrip1l is unaltered, whereas the ciliary amount of Nphp5 is reduced. This leads to down-regulation of ciliary gating in Cep290−/− cells. Restoration of the Cep290 amount in Rpgrip1l−/− cells via transfection of full-length Cep290 rescues ciliary gating defects. We propose that the rescue of ciliary gating in transfected Rpgrip1l−/− cells is mediated by a restored ciliary amount of Nphp5.
increased ciliary length. Remarkably, we previously showed that Rpgrip1l deficiency leads to a reduced autophagic activity and that the treatment of Rpgrip1l−/− MEFs with autophagy activators rescues cilia length (Struchtrup et al., 2018). For this reason, Rpgrip1l might regulate cilia length by controlling autophagy. Investigating the complex mechanisms of ciliogenesis will be a major task for the future of cilia research.

The role of TZ proteins in ciliary gating

Interestingly, Garcia-Gonzalo et al. revealed that the loss of the TZ gatekeeper protein Tmem67 diminishes the ciliary amount of Arl13b and Ac3 but the ciliary amount of Smo remains normal (Gerhardt et al., 2011). The lack of Cep290 and Nphp5 in RPE-1 cells results in a reduced ciliary amount of Smo (Barbelanne et al., 2015b). Since the amount of Cep290 is reduced in Rpgrip1l−/− MEFs (Wiegering et al., 2018a), the expectation would be that the amount of Smo was decreased in these MEFs. However, these findings have been made in different cell types making it possible that the ciliary gating function of these proteins might be cell type specific. The analysis of this hypothesis is an exciting subject of future studies which would shed further light on the ciliary gating function of the TZ.

Many ciliopathies can be attributed to mutations in genes encoding TZ proteins (Hildebrandt et al., 2011; Czarniecki and Shah, 2012). For this reason, the assembly and function of the TZ is a hot topic in biomedical research. A lot of proteins participate in TZ assembly and/or function as ciliary gatekeepers at the TZ (Craige topic in biomedical research. A lot of proteins participate in TZ proteins (Hildebrandt et al., 2011) demonstrating the existence of a specificity between the gatekeeper proteins and the proteins which are allowed to cross the TZ. In Rpgrip1l−/− MEFs, the ciliary amount of Smo is also unaltered (Gerhardt et al., 2011; Aubusson-Fleury et al., 2012; Cevik et al., 2016; Vieillard et al., 2013; Wang et al., 2013; Awata et al., 2014; Basiri et al., 2014; Klinger et al., 2014; Tuz et al., 2014; Bachmann-Gagescu et al., 2015; Barbelanne et al., 2015b; Damerla et al., 2015; Roberson et al., 2015; Yee et al., 2015; Lambacher et al., 2016; Li et al., 2016; Pratt et al., 2016; Slaats et al., 2016; Vieillard et al., 2016; Wei et al., 2016; Dyson et al., 2017; Lu et al., 2017; Schou et al., 2017; Shi et al., 2017; Takao et al., 2017; Jensen et al., 2018; Scheidel and Blacque, 2018; Wiegering et al., 2018a; Jack et al., 2019; Lapart et al., 2019; Lewis et al., 2019). However, the relationships between these proteins and hence the mechanisms underlying ciliary gating at the TZ remain largely elusive. Recently, we showed that Rpgrip1l represents a central factor in vertebrate TZ assembly (Wiegering et al., 2018a). Our current study reveals that Rpgrip1l also regulates ciliary gating by ensuring the proper amount of Cep290 at the vertebrate TZ. Combining our results with previous findings, we suggest a protein hierarchy regulating ciliary gating in which Csppl1, Rpgrip1l, Cep290, and Nphp5 are involved. Our work is an important piece of a puzzle depicting this fundamental ciliary process. The completion of this puzzle will be one of the most important tasks of cilia research in the next few years.

MATERIALS AND METHODS

Request a protocol through Bio-protocol.

See Table 1 for key resources.

Cell lines

We used two different cell lines in this study. NIH3T3 cells (#ACC59) and HEK293 cells (#ACC35) were both purchased by the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ).

Cells were grown in DMEM supplemented with 10% fetal calf serum (FCS), 1/100 (vol/vol) L-glutamine (Life Technologies), 1/100 (vol/vol) sodium pyruvate (Life Technologies), 1/100 (vol/vol) nonessential amino acids (Life Technologies), and 1/100 (vol/vol) pen/strep (Life Technologies) at 37°C and 5% CO2. The following clones were used: Rpgrip1l−/− NIH3T3 cells (clone 10-61), Cep290−/− NIH3T3 cells (clones 39-10, 39-51, 39-53) (Wiegering et al., 2018a), Nphp1−/− NIH3T3 cells (clone 21-21) (Wiegering et al., 2018a), Invs−/− NIH3T3 cells (clones 48-7, 48-20) (Wiegering et al., 2018a), and RGRIP1L−/− HEK293 cells (clone 1-7) (Wiegering et al., 2018a).

Primary cell culture

We isolated MEFs from single mouse embryos (male and female) at embryonic stage (E) 12.5 after standard procedures. MEFs were grown in DMEM supplemented with 10% FCS, 1/100 (vol/vol) L-glutamine (Life Technologies), 1/100 (vol/vol) sodium pyruvate (Life Technologies), 1/100 (vol/vol) nonessential amino acids (Life Technologies), and 1/100 (vol/vol) pen/strep (Life Technologies) at 37°C and 5% CO2. The following mutant mice were used: Rpgrip1l−/− mutant mice on a C3H-background (Vierkotten et al., 2007) and Nphp4−/− mutant mice on a C57BL/6J-background (Wiegering et al., 2018a).

Cell culture, transfection, and drug treatment

Ciliogenesis in confluent-grown MEFs and NIH3T3 cells was induced by serum starvation (0.5% FCS) for at least 24 h. For DNA transfection, Lipofectamin 3000 (Invitrogen) was used following the manufacturer's guidelines. Appropriate empty vectors were used as transfection control (TF-Ctrl). NIH3T3 cells were treated with 20 μM eupatilin (#5ML1689; Sigma-Aldrich) or DMSO as a control solvent for 24 h.

Antibodies and plasmids

Cells were immunolabeled with primary antibodies targeting Arl13b (#17711-1-AP; Proteintech and #75-287; Antibodies Incorporated), Cep290 (#ab84870; Abcam), Csppl1 (#11931-1-AP; Proteintech), Flag (#F7425; Sigma-Aldrich; Myc (#sc-789; Santa Cruz Biotechnology), Nphp5 (#15747-1-AP; Proteintech), Sept7 (#11397-1-AP; Proteintech), Sept7 (#13818-1-AP; Proteintech), Sestr3 (#20696-1-AP; Proteintech), RPGRIP1L (#PA3-207; Pierce Biotechnology and #11617; Santa Cruz Biotechnology), acetylated α-tubulin (#53-23950; Santa Cruz Biotechnology; #T-6793; Sigma-Aldrich), and γ-tubulin (#sc-7396; Santa Cruz Biotechnology). The generation of the polyclonal antibody against Rpgrip1l was described formerly (Vierkotten et al., 2007).

The following plasmids were used: pMyc-mNphp1 (kindly provided by Sophie Saunier), eGFP-mCEP290 (kindly provided by Hemant Khanna), and pFlag-mCep290 (#27381; Addgene), PMC-mNphp1 encodes for the murine full-length Nphp1 protein fused to a GFP-tag (vector: CMV), GFP-hCEP290 encodes for the human full-length Cep290 protein fused to a GFP-tag (vector: CMV), and pFlag-mCep290 encodes for the murine full-length Cep290 protein fused to a Flag-tag (vector: CMV2).

CRISPR/Cas9-mediated gene inactivation

Inactivation of mouse Rpgrip1l in NIH3T3 cells was performed as previously described (Wiegering et al., 2018a). We choose a target site which is located in exon3 of the gene (Supplemental Figure S2). After inactivation and single-cell cloning, eight clones, which on RFLP analysis appeared to have lost the diagnostic Eag sequence, were further analyzed. To establish the genotype, individual alleles were cloned and sequenced (Supplemental Figure S2).
### Table 1: Key resources.

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Sequences of the target site and primer pairs used to amplify the targeted region are as follows:

Rpgrip1l − T3: CTCGAGTTAACACCGGCCGCCGG
Rpgrip1l-T3b-for: GAATGGCCACCAAGTTAATACGGCTAG
Rpgrip1l-T3b-rev: CTTCAGGATCTGACAGAGAGCAAGCCTC.

Off-target analyses

Off-target analyses were performed by RFLP analyses as previously described (Wieginger et al., 2018a). For the on-target Rpgrip1l−T3 there exist no off-targets carrying one, two, or three mismatches. From the remaining four-mismatch off-targets, we tested the top-three ranking sites (Hsu et al., 2013) (crispr.mit.edu/) on the DNAs from the same eight-set clones analyzed for targeting of the on-target. In summary, we did not detect any mutations (unpublished data).

Sequences of the off-target sites and primer pairs used for amplification are as follows:

T3 off-target-1: CACGAGTCAGCACCGGCCACTGG
T3 off-1 for: CTGTCAGGTTTCCCAGTGTGCAG
T3 off-1 rev: CTCTCAGCTCCTTTTAGGTCTCCAG
T3 off-target-2: CTCTACTGAACAACGGCCGCAGG
T3 off-2 for: ATCCAGCCAAACCGCCGGCAG
T3 off-2 rev: GGTTTGTCTCTGTCCTGACATGTCAC
T3 off-target-3: ATCCAGTTGACACCGGCCTCTGG
T3 off-3 for: GTCTCCTTCAGACCCACTGAAGTG
T3 off-3 rev: GTCCCAGGAAGCCAGGCTTGTG.

The following restriction enzymes were used: BsmI (T3 off-target-1), EagI (T3 off-target-2), and BstII (T3 off-target-1).

Image acquisition

Image acquisition and data analysis were carried out at room temperature using a Zeiss Imager.A2 microscope, 100×, NA 1.46 oil immersion objective lens (Carl Zeiss AG), a monochrome charge-coupled device camera (AxioCam MRm, Carl Zeiss AG), and the AxioVision Rel. 4.8 software package (Carl Zeiss AG), or a TI-Eclipse Nikon inverted microscope, 100×, 1.49 oil immersion objective lens coupled with a 95B Prime 22 mm Photometrics sCMOS camera. A Nikon fluorescent lamp and a quadriband dichroic block were used to detect blue, green, and red fluorescence. The acquisition software NIS was used. Three single-plane images per cilium were obtained in an 8-bit grayscale modus respectively covering the specific spectrum of the used fluorochrome. Appropriate anti-mouse, anti-rabbit and anti-goat Alexa405, Cy3, Alexa594, and Alexa488 antibodies were used as fluorochromes.

Immunofluorescence

For immunofluorescence on MEFs, NIH3T3 cells, and HEK293 cells, cells were plated on coverslips until confluency. MEFs and NIH3T3 cells were serum-starved for at least 24 h. Cells were fixed with 4% paraformaldehyde (for staining with the antibodies to Cep290, Rpgrip1l, Flag, Myc, Sept7, and Arl13b) or methanol (for staining with the antibodies to Cspp1, Nphp5, Sept2, and Sstr3). Fixed cells were rinsed three times with phosphate-buffered saline (PBS), followed by a permeabilization step with PBS/0.5% Triton X-100 for 10 min. The samples were rinsed three times with PBS. Samples were incubated for at least 10 min at room temperature in PBST (PBS/0.1% Triton X-100) containing 10% donkey serum or 10% normal goat serum. Diluted primary antibodies (in blocking solution) were incubated overnight at 4°C. After three washing steps with PBST, incubation with fluorescent secondary antibody (diluted in

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**TABLE 1: Key resources. Continued**
Western blotting
Whole-cell lysates were obtained by lysis with radioimmunoprecipitation buffer (150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 0.1% sodium deoxycholate, and 1 mM EDTA). Protein content was measured by the Bradford method, and samples were normalized. Total protein (20 μg) was separated by SDS-PAGE on polyacrylamide gels (#456-1093; Bio-Rad Laboratories) and transferred to a PVDF membrane (#162-0176; Bio-Rad). The membrane was probed with antibodies against Flag (#F7425; Sigma-Aldrich), GFP (#A-6455; Thermo Fisher Scientific), and Myc (#sc-789; Santa Cruz Biotechnology). Anti-Gapdh (#ab9485; Abcam) antibody was used as a loading control. Proteins were detected with secondary antibodies conjugated to horseradish peroxidase (RPN4201 and RP4301) and the SuperSignal West Pico PLUS detection kit (#34580; Thermo Fisher Scientific). Visualization of protein bands was realized by GBbox (SYNGENE).

Quantification and presentation
Ciliary protein staining and protein bands intensity were quantified using ImageJ (National Institutes of Health). Intensity measurement of proteins based on immunofluorescence staining was performed as described before (Garcia-Gonzalo et al., 2011; Garcia-Gonzalo et al., 2015; Gerhardt et al., 2015; Roberson et al., 2015; Yee et al., 2015; Struchtrup et al., 2018; Wiegeler et al., 2018a). Triplets of 8-bit single-plane grayscale images were merged via ImageJ. The merged images were not further processed and the signal intensities were measured. The ciliary length has to be taken into account while quantifying the ciliary amount of Arl13b and Str3 in different genotypes. Therefore we used the area marked by acetylated α-tubulin as a reference and quantified the average pixel intensity of the Arl13 and Str3 staining. For all other ciliary protein intensities (Cep290, GFP, Flag, Csp1p, Myc, Nphp5, Rpgrp11, Sept2, and Sept7), we selected the region labeled by γ-tubulin (for BB proteins) or the area in between the γ-tubulin staining and the proximal part of the acetylated α-tubulin staining and measured the total pixel intensity. To exclude unspecified staining from the measurements, we subtracted the mean value of the average pixel intensity (in the case of Arl13b and Str3) or of the total pixel intensity (all other ciliary proteins) of three neighboring regions free from specific staining.

Representative images were processed after quantification of ciliary protein staining was completed. The images were processed by means of background subtraction and contrast settings via Adobe Photoshop CS2.

Statistical analysis
Data are presented as mean ± SEM. Two-tailed t test with Welch’s correction was performed for all data in which two datasets were compared. Analysis of variance (ANOVA) and Tukey honest significance difference (HSD) tests were used for all data in which more than two datasets were compared. *P < 0.05 was considered to be statistically significant, **P < 0.01 was defined as statistically very significant, and ***P < 0.001 was noted as statistically highly significant. Sample sizes are indicated in the figure legends and the power of statistical tests was verified via post-hoc power calculations.

All statistical data analysis and graph illustrations were performed by using GraphPad Prism (GraphPad Software) and the Post-hoc Power Calculator (https://clincalc.com/Stats/).

DATA AND CODE AVAILABILITY
This study did not generate datasets.

ACKNOWLEDGMENTS
The authors thank Matias Zurbriggen and Leonie-Alexa Koch for their generous help to enable the continuation of the study. Moreover, we are grateful to Sophie Saunier for providing the Myc-Nphp1 construct. We thank the cell imaging facility of the IBPS (Institut de Biologie Paris-Seine FR3631, Sorbonne Université, CNRS, Paris, France) for their technical assistance. This work was funded by the Fondation ARC pour la Recherche sur le Cancer (Project ARC PJA 20171206591 to S.S.M.), the Fondation pour la Recherche Médicale (Equipe FRM EQU201903007943 to S.S.M.), and the German Research Foundation (DFG; Grant No. WI 5451/1-1 to A.W.).

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