Mutations in a Guanylate Cyclase GCY-35/GCY-36 Modify Bardet-Biedl Syndrome–Associated Phenotypes in Caenorhabditis elegans

Calvin A. Mok1,2,3, Michael P. Healey4, Tanvi Shekhar1, Michel R. Leroux4, Elise Héon1,3,5, Mei Zhen2,3,5*

1 The Program in Genetics and Genome Biology, The Hospital for Sick Children, Toronto, Canada, 2 Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada, 3 Institute of Medical Science, University of Toronto, Toronto, Canada, 4 Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, Canada, 5 Department of Molecular Genetics, University of Toronto, Toronto, Canada

Abstract

Ciliopathies are pleiotropic and genetically heterogeneous disorders caused by defective development and function of the primary cilium. Bardet-Biedl syndrome (BBS) proteins localize to the base of cilia and undergo intraflagellar transport, and the loss of their functions leads to a multisystemic ciliopathy. Here we report the identification of mutations in guanylate cyclases (GCYs) as modifiers of Caenorhabditis elegans bbs endophenotypes. The loss of GCY-35 or GCY-36 results in suppression of the small body size, developmental delay, and exploration defects exhibited by multiple bbs mutants. Moreover, an effector of cGMP signalling, a cGMP-dependent protein kinase, EGL-4, also modifies bbs mutant defects. We propose that a misregulation of cGMP signalling, which underlies developmental and some behavioural defects of C. elegans bbs mutants, may also contribute to some BBS features in other organisms.

Introduction

The cilium plays diverse cellular functions in metazoans which include imparting motility, enabling sensory processes and regulating the activity of cell signalling pathways during development [1]. The biogenesis and maintenance of this evolutionarily conserved organelle relies on intraflagellar transport (IFT) – the bidirectional transportation of diverse cargo proteins along the microtubule-based axoneme. Defective IFT or ciliary dysfunction result in ciliopathies, a growing class of pleiotropic human diseases with overlapping clinical features, some being of significant morbidity [2]. Bardet-Biedl syndrome (BBS, OMIM 209900) is an autosomal recessive and genetically heterogeneous ciliopathy with hallmarks of clinical features that include photoreceptor degeneration, renal anomalies and obesity [15–16]. Additionally, these models led to the identification of new features such as neural tube closure defects [17], anosmia [18], and behavioural, mechano- and thermosensory deficits [14] that expanded the diagnostic features of human ciliopathies. Morpholino-mediated knockdown of bbs in zebrafish led to developmental phenotypes such as dorsal thinning, poor somitic definition and Kopffer’s Vesicle malformation [19–21], while defects characteristic of ciliopathy such as delayed retrograde melanosome transport [20] and vision impairment [22] also manifested.

In addition to a role in sensory transduction, the primary cilium functions as a signalling ‘apparatus’ to regulate development [1]. For example, IFT-dependent localization of Sonic Hedgehog (Shh) receptors to primary cilia is required for Shh signalling [23–24]. Disrupting IFT components IFT172, TG737/Polaris and the motor KIF3A in the mouse resulted in phenotypes typical of Shh mutants [25]. Similarly, defective planar cell polarity (PCP) signalling [17,26] and/or aberrant Wnt signalling [27–28] were associated with the inactivation of BBS, Polaris or KIF3A components. These, and others studies [29–30] suggest that the cilium may modulate multiple signalling pathways in a tissue-specific manner. Aberrant PCP, Shh and Wnt signalling have been implicated in underlying a number of ciliopathy features, such as neural tube closure, polydactyly and obesity [17,22,31–32]. The pathology of other features such as photoreceptor degeneration, remains largely unexplained, indicating the presence of unidentified cellular processes that are regulated by the cilium.

Murine Bbs mutants recapitulate several human BBS features including photoreceptor degeneration, renal anomalies and obesity [15–16]. Additionally, these models led to the identification of new features such as neural tube closure defects [17], anosmia [18], and behavioural, mechano- and thermosensory deficits [14] that expanded the diagnostic features of human ciliopathies. Morpholino-mediated knockdown of bbs in zebrafish led to developmental phenotypes such as dorsal thinning, poor somitic definition and Kupffer’s Vesicle malformation [19–21], while defects characteristic of ciliopathy such as delayed retrograde melanosome transport [20] and vision impairment [22] also manifested.

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Persisted throughout adulthood (Figure 1B and Figure S1). In animals. Defects were visible during early larval stages and bbs-7 developmental timing.

Reduced body length by we identified three novel functional deficit in the sensory cilia. In addition to sensory defects, sensory neurons (Figure 1A), confirming a common structural and mutants exhibited a failure in the uptake of a lipophilic dye DiI by C. elegans bbs signalling events in present study, in order to identify additional cilium-regulated size regulation, however, remain to be fully elucidated. In the conclusion, this study reveals a non-cell autonomous role for sensory cilia in regulating cGMP signalling during development. We propose that aberrant cGMP signalling, essential for a number of cellular processes, may also contribute to some ciliopathy features in other systems.

C. elegans BBS orthologues are exclusively expressed by 60 ciliated neurons. Localizing at the base of cilia, they undergo active IFT, and their absence results in the destabilization of IFT and sensory defects [8]. C. elegans sensory neurons play key roles in multiple developmental processes [33]. Some chemosensory mutants exhibit a reduced body size [33], indicating that sensory function may influence this developmental process. Another key regulator for body size is the cGMP-dependent protein kinase (PKG) EGL-4 [33–36]; a loss of EGL-4 function leads to increased body size that is genetically epistatic to that of chemosensory mutants [33]. The mechanisms for sensory neuron-mediated body size regulation, however, remain to be fully elucidated. In the present study, in order to identify additional cilium-regulated signalling events in C. elegans, we carried out the phenotypic characterization of bbs mutant animals, and identified genetic modifiers that associate aberrant cGMP signalling with a subset of bbs features.

Results

C. elegans bbs mutants share ciliary defects, reduced body size, delayed developmental timing, and reduced roaming

We performed a thorough phenotypic and behavioural assessment of severe or complete loss-of-function (f) mutants for the C. elegans bbs-1, -2, -7, -9 and -9 genes (Table S1). Consistent with previous reports on bbs-7 and -9 [13], all examined bbs mutants exhibited a failure in the uptake of a lipophilic dye DiI by sensory neurons (Figure 1A), confirming a common structural and functional deficit in the sensory cilia. In addition to sensory defects, we identified three novel bbs-associated phenotypes: decreased body size, altered dwelling/exploration behaviour and delayed developmental timing.

Despite grossly normal body morphology, bbs mutants shared a reduced body length by ~11–28% when compared to wild-type animals. Defects were visible during early larval stages and persisted throughout adulthood (Figure 1B and Figure S1). In these analyses, we defined a 3.5% or greater difference as biologically relevant in body size change, as this was the upper range for the coefficient of variance in young adult wild-type populations. The reduced body length is caused by the loss of BBS function, as it was fully rescued by introducing a wild-type genomic or cDNA copy of bbs into the respective mutants (Figure 1C). A decrease in body width was also characteristic of bbs mutants (Figure 1D). By DAPI staining of nuclei we did not observe differences in tissue and cell numbers between wild-type and bbs-7 animals (data not shown). The overall decrease in body size is thus best attributed to a smaller, averaged cell size in bbs mutants.

bbs double and triple mutants exhibited smaller body sizes that were no more severe than the smallest bbs single mutant. Although small differences between the body length of bbs single, double and triple mutant strains were observed as animals aged, the effects, however, were not additive (Figure 1B), consistent with BBS proteins functioning in the same biological processes to regulate ciliary development and function [6–7,37].

We further examined the body size of a number of IFT mutants, including che-2, -3, and -11, as well as osm-3, -5, -6 and klp-11, all of which display defects to cilia structure and abnormal dye filling (dyf). Only some exhibited decreases in body size; among them, che-11 mutants exhibited the most significant decrease (by 11% in young adulthood), but not as severely as in age-matched bbs mutants (Figure S2A). Notably, a loss of the IFT motors, KLP-11 and OSM-3 kinesins, and CHE-3 dynein had little or no effect on body size. Therefore while sensory neurons affect body size, the dyf phenotype, caused by defective IFT transport, is not indicative of severe body size defects. The BBSome, exclusively expressed in ciliated sensory neurons, has a greater influence in the regulation of body size, indicating a role beyond bridging IFT motors for BBS proteins in these neurons.

C. elegans exhibits a defined developmental time course [38]. Multiple bbs mutant strains exhibited slower larval development (Figure 1E), resulting in a 2.3–6.2 hour delay between the first (L1) and last (L4) larval stage. During foraging, C. elegans exhibits a combination of dwelling and exploration/roaming behaviours that are altered in some chemosensory defective mutants [33]. Multiple bbs mutants showed a 56% to 76% decrease in overall movement, or roaming (Figure 1F) when compared to wild-type animals. This behavioural change does not reflect a general loss of locomotor activity, as bbs mutant animals exhibit normal locomotion during roaming. These additional phenotypes support a notion that the C. elegans cilia regulate cellular processes in addition to taxis behaviours.

The loss of function of a guanylate cyclase subunit GCY-35 rescues a subset of endophenotypes in bbs mutants

Unlike all other bbs strains, MT3645 bbs-7(n1606) (received from the Caenorhabditis Genetics Center) displayed body size, roaming behaviour and developmental timing characteristics of wild-type animals. Upon genetic outcrossing, we re-isolated a homozygous bbs-7(n1606) strain that exhibited phenotypes characteristic of other bbs mutants. These defects were fully rescued by the expression of the wild-type genomic copy of bbs-7 (Figure 1D). We concluded through genetic analyses that a single modifying locus from the original MT3645 strain rescued a subset of the bbs-7 mutant endophenotypes (Figure 2).

We mapped this modifier allele, hp433, to gcy-35 (Materials and Methods), a gene encoding the subunit of a soluble guanylate cyclase (sGC). sGC proteins are composed of a heme/NO binding (HNOB), a heme/NO binding associated (HNOBA), and a GC catalytic domain (reviewed in [39]). They are heterodimeric
Figure 1. Multiple endophenotypes exhibited by bbs mutants. (A) Dil uptake of bbs mutants was reduced compared to wild-type and transgenic bbs mutants carrying genomic DNA fragments for respective bbs genes. Fisher’s Exact test, *** p<0.001. (B) The body length of young adult bbs single, double and triple mutants was smaller in comparison to wild-type animals. ANOVA with Tukey, *** p<0.001, n≥20. Data represent mean ± SD normalized against wild-type body length. (C) The body length defects of bbs mutants were rescued by the expression of wild-type bbs genes (+) compared to their non-transgenic siblings (−). ANOVA with Tukey, *** p<0.001, n≥15. (D) The body width of bbs mutants was also decreased in comparison to wild-type animals. ANOVA with Tukey, *** p<0.001, n≥20. (E) Developmental timing (from L1 to L4 stage) was increased for bbs mutants in comparison to wild-type animals. ANOVA with Tukey, *** p<0.001, n=50, N=10 replicates. Data represent mean (hours) ± SD. (F) bbs mutants roamed less compared to wild-type animals. Kruskal-Wallis with Dunn’s, *** p<0.001, n≥25, N=2 replicates. Data represent mean squares roamed ± SEM. For bbs-7 dataset, the ok1357 deletion allele was used unless otherwise noted. doi:10.1371/journal.pgen.1002335.g001
complexes consisting of α and β subunits to catalyze the conversion of GTP to cGMP. hpfl3 results in a frame-shift in the coding sequence and a premature termination codon in the HN0bA domain, causing a truncation of the GC catalytic domain (Figure 2A). We also saw that a deletion allele in the GC domain of gcy-35, ak769, functions as a recessive suppressor of bbs-7 mutants (Figure 2A and 2B).

While both gcy-35(lf) alleles exhibit similar body length to that of wild-type animals, they suppressed the significant body length defects of multiple bbs mutants (Figure 2B). In contrast, gcy-35(+/+)/bbs-7 mutants showed little or no improvement to body length (Figure S2B). The consistent suppression observed in bbs mutant animals advocated strongly for the further investigation of gcy-35(lf) as an epistatic suppressor of bbs-mediated phenotypes. gcy-35 also modified the bbs endophenotypes in developmental timing and roaming. Both the developmental timing from the first (L1) to the last (L4) stages, and roaming scores of gcy-35 mutants were comparable to that of wild-type (Figure 2C and 2D). The developmental timing of gcy-35(bbs-7) and gcy-35(bbs-2) mutants was identical to that of wild-type animals, whereas gcy-35(bbs-8) animals showed a partial improvement over that of bbs-8 animals (Figure 2C). Roaming defects of bbs-2, bbs-7, and bbs-8 mutants were partially suppressed by gcy-35 (Figure 2D).

Other bbs phenotypes, such as shortened cilia and defective Dil uptake (Figure 2E; and data not shown), were not rescued by gcy-35(lf) mutants. These results suggest that the ciliary structures remain impaired in gcy-35(bbs) mutants, and that the cellular pathways regulating body size, developmental timing and roaming behaviours either function genetically downstream of, or differ from those involved in sensation.

The GCY-35/GCY-36 sGC complex influences the body size of bbs mutants through a subset of oxygen sensing body cavity neurons

GCY-35 and its partner GCY-36 form a heterodimeric sGC that modulates C. elegans behaviour in response to ambient oxygen concentrations [40–41]. gcy-35(bds66) (lf) mutants [40] rescued the body size defect of bbs-7 mutants (Figure 2B). Moreover, gcy-35; gcy-36(bds66) animals exhibited a body size different from either gcy-35(bds66) or gcy-36(bds66) mutant (Figure 2B), consistent with GCY-35 and GCY-36 also functioning as a heterodimeric sGC to regulate body size.

We examined in which neurons this sGC influences body size using bbs mutants. Both GCY-35 and GCY-36 are expressed in the ciliated body cavity sensory neurons AQR and PQR, and a non-ciliated body cavity neuron URX; GCY-35 is additionally expressed in the non-ciliated ALN, PLN, and SDQ neurons [41]. AQR, PQR and URX expression of wild-type GCY-35 in gcy-35(bbs-7) animals reverted body size similarly to that of bbs-7 mutants, whereas GCY-35 expression in ALN, PLN, and SDQ had no effect (Figure 3A). Similarly, the expression of a GFP-tagged GCY-36 in AQR, PQR and URX neurons in bbs-7 gcy-35(G0) animals was also sufficient to revert body size close to that of bbs-7 mutants (Figure 3A). This suggests a specific requirement of GCY-35 and GCY-36 in body cavity neurons to regulate body size.

Among them, URX appears to be the most essential neuron, as restoring GCY-35 in URX (and other neurons not normally expressing gcy-35/gcy-36) by an exogenous promoter showed a partial, but significant reversion of the body size in gcy-35 bbs-7 animals (Figure 3A). Similarly, GCY-36 is most essential in URX: we conducted a mosaic analysis of bbs-7 gcy-35 animals carrying a functional GFP::GCY-36 transgene, and we observed expression of GFP::GCY-36 in URX of all rescued animals (Figure 3B). URX expression of the GCY-35/GCY-36 sGC is therefore essential, although not fully sufficient, to regulate the body size of bbs mutants.

Finally, if the body cavity neurons contribute to the small size phenotype of bbs mutants, their ablation by the transgene qaIs2241[pgy-36::egl-4] [42] in a bbs background should also have a rescuing effect. While both qaIs2241 and gcy-35 qaIs2241 animals exhibited normal body sizes, bbs-7 qaIs2241 mutants showed a significantly increased body size when compared to bbs-7 animals (Figure 3C), further supporting that these neurons modulate the body size of bbs mutants.

The expression and localization of GFP::GCY-35 and GFP::GCY-36 are grossly normal in bbs mutants

Our genetic analyses indicate that BBS proteins negatively regulate sGC-mediated signalling activity. We examined if bbs mutants exhibit an elevated expression and/or expanded localization of sGC in these neurons. As reported [40], a functional GFP::GCY-36 localized largely to the soma and along the dendrites of AQR, PQR and URX. We did not observe significant changes in its expression or localization in bbs mutants (Figure S3), nor did we see a loss of ciliary localization as reported for the loss of a putative isopenylation signal at the C-terminus [40]. bbs mutations did not perturb, at a gross level, the expression and localization of GFP::GCY-35 either (Figure S3). We did observe a high degree of morphological variability in the dendritic endings of URX, AQR, and PQR neurons as previously reported [43] in both wild-type and bbs mutants. However, given the morphological variability, we cannot exclude the possibility of subtle alterations in the length of bbs mutants. Therefore, while AQR, PQR and URX neurons regulate the body size through a process that involves sGC activity, it does not appear to result directly from altered sGC protein level or subcellular localization.

The cGMP-dependent protein kinase (PKG) EGL-4 is a sGC effector in body size regulation

cGMP is a key secondary messenger (reviewed in [44]). In C. elegans, cGMP activates a heteromeric cGMP-gated ion channel TAX-2/TAX-4 for oxygen sensation and other sensory processes [45]. cGMP also activates a PKG, EGL-4, to regulate olfactory adaptation, life span, behavioural states and body size [33–34,46–47]. Specifically, egf-4(lf) mutants exhibit a large body size, and are epistatic to the reduced body size and roaming behaviour of some sensory mutants; whereas a constitutively active, gain-of-function (gf) egf-4 mutation, causes a small body size [36]. The shared effects of bbs and egf-4 suggested that EGL-4 could be a downstream effector of sGcs in body size regulation.
We examined the *egl-4* mutants (Figure 4) for their genetic interactions with *bbs-7* and *gcy-35* mutants. *egl-4(lf)* alleles were epistatic to *bbs-7*, *gcy-35*, and *bbs-7;gcy-35* for body size and developmental timing (Figure 4B and 4C). *egl-4(gf)* also exhibited an epistatic effect to *bbs-7* and *bbs-7;gcy-35* mutants, although *egl-4(gf);bbs-7* and *egl-4(gf);bbs-7;gcy-35* mutants may exhibit a slightly
more severe phenotype than egl-4(gf) that was only distinguished by statistical analyses (Figure 4B). By contrast, if mutants for another cGMP effector, the cGMP-gated cation channel subunit TAX-2 and TAX-4, did not exert body size suppression for bbs-7 (Figure S4). Moreover, some TGF-β signalling mutants exhibited altered body size [48–50]. While EGL-4 was proposed to function genetically downstream of TGF-β signalling [33], body size defects of several TGF-β mutants exhibited additive effects in bbs-7 or bbs-7 gcy-35 backgrounds (Figure 4D), suggesting that TGF-β signalling and BBS-mediated body size regulation likely operates in an additive or parallel manner. Taken together, these results confirm a specific genetic relationship between bbs-7, gcy-35 and egl-4 for cilia-mediated body size regulation and developmental timing.

To further test if the body size influence of GCY-35/GCY-36 sGC functions through EGL-4, we expressed egl-4 cDNA harbouring the ad450(gf) mutation in AQR, PQR and URX neurons of gcy-35;bbs-7 animals. If GCY-35 regulates body size through activating EGL-4, a constitutively activated EGL-4(ad450) in these neurons should abrogate the body size suppression by the loss of gcy-35. Indeed, this transgene reverted animals to a bbs-7-like body size (Figure 4E). Wild-type animals expressing the same transgene did not exhibit changes in body size (Figure 4E). Not only is this result consistent with elevated cGMP signalling contributing to the reduced body size in bbs mutants, it further suggests that GCY-35/GCY-36 regulates body size through EGL-4 in the body cavity neurons.

BBS proteins are required in multiple sensory neurons to regulate body size

To investigate whether ciliary functions regulate body size strictly through body cavity neurons like GCY-35/GCY-36, we expressed a functional BBS-7 or GFP::BBS-2 in the AQR, PQR and URX neurons of bbs-7 or bbs-2 mutants, respectively. The small body size of respective bbs mutants was not rescued (Figure 5A). We did, however, observe a complete rescue of the body size with a pan-neuronally expressed GFP::BBS-2 in bbs-2 mutants (Figure 5A). Moreover, we observed a full or partial rescue of the body size defects when a functional BBS-7 was expressed in at least two non-overlapping groups of sensory neurons AWB, AWC, ADF, ADR, ASH and PHA or ADL, ADF, ASH, PHA and PHB (Figure 5A). All these neurons have sensory cilia exposed to outside of the body cavity. Therefore, while the GCY-35/GCY-36 sGC functions through body cavity neurons to regulate body size, restoring ciliary function in these neurons alone does not sufficiently reduce cGMP signalling to restore body size. Alternatively, other sensory neurons that input onto the body cavity neurons, may serve to regulate body size. Regardless, the observation that BBS proteins can influence body size through different groups of sensory neurons is reminiscent of the previously reported observation that the body size of egl-4(gf) mutants could be rescued by restoring EGL-4 expression in non-overlapping sets of sensory neurons [33].

**Figure 4.** EGL-4 is an effector of GCY-35/GCY-36 in influencing body length and developmental timing. (A) A schematic of EGL-4 showing allelic information on the ad450sd (gain-of-function) and m478 (loss-of-function). egl-4(gf) and egl-4(lf) are epistatic to bbs-7(ok1351) (gray bars), gcy-35(hp443) (diagonal striped bars) and gcy-35;bbs-7 (dark bars) for both body size (B) and developmental timing (C). ANOVA with Tukey, *** p < 0.001; ns = p > 0.05 or body length difference < 3.5%. For body length, data represent mean ± SD normalized against wild-type body length, n = 20. For developmental timing, data represent mean (hours) ± SD, n = 50, N = 10 replicates. (D) Loss of function mutations in the TGF-β pathway (BMP-S/abl-1 and lon-2, white bars) had an additive effect on regulating body size in conjunction with bbs mutants (light bars), while being unaffected by the loss of gcy-35(ok769) (dark bars). ANOVA with Tukey, *** p < 0.001; ns = p > 0.05, n = 20. Data represent mean ± SD normalized against wild-type body length. (E) The transgenic expression (+, light bars) of egl-4(gf) in body cavity neurons reverted the body size suppression in gcy-35;bbs-7 in comparison to non-transgenic siblings (−, dark bars). EGL-4(gf) expression within body cavity neurons did not affect the body size of wild-type transgenic controls. ANOVA with Tukey, *** p < 0.001; ns = p > 0.05, n = 20. Data represent mean ± SD normalized against wild-type body length. doi:10.1371/journal.pgen.1002335.g004

**GCY-35/GCY-36 acts as a predominant effector in cilia-mediated body size regulation**

That multiple, non-overlapping and non-body cavity ciliated sensory neurons (CSNs) regulate body size suggests a cumulative effect on cGMP signalling-mediated body size regulation by multiple sensory neurons, or, a predominating effect by body cavity neurons in cilia-mediated body size regulation through GCY-35/GCY-36. The first model predicts that additional GCSs in other sensory neurons would epistatically suppress body size. The *C. elegans* genome encodes 7 sGCs and 27 receptor-like GCSs (rGCSs) [51]. Single loss-of-function mutations in other sGCs, GCY-31, 32, 33, 34 and 37 all failed to alter bbs-7 body size defects (Figure S5A and data not shown). Of 16 rGCS mutants tested, four exhibited modifying effects, two very mildly suppressing and two significantly exacerbating the smaller body size of bbs-7 animals, but none suppressed bbs-7 mutant phenotypes comparable to gcy-35/gcy-36 (Figure 5B and Figure S5A). We examined the effect of several double and triple rGCS mutants on bbs-7 body size to explore the possibility that multiple rGCS function redundantly [52] but we did not observe obvious suppression effect in these additional mutants (Figure S5A).

We further examined the combinational effect of gcy-32 and other rGCS mutants on bbs-7, including a mildly suppressing allele (gcy-4(tm1653)), two exacerbating alleles (gcy-7(tm901) and gcy-7(ok2538)) and three “neutral” alleles (gcy-23(ok797), gcy-28(tm2411) and gcy-25(tm4300)). We did not observe a significant body size improvement between gcy-35;bbs-7 and these triple mutants (Figure S3B). Therefore with the caveat that we have not exhausted the examination of all single or combinational GC mutants, GCY-35/GCY-36, through the body cavity neurons, act uniquely as a predominating effector for BBS-mediated body size regulation.

**Discussion**

In the present study, we show that *C. elegans* bbs mutants exhibit reduced body length, delayed development and altered roaming pattern, in addition to known sensory defects. These endophenotypes depend, fully or in part, on the GCY-35/GCY-36 sGC complex, through its effector EGL-4 PKG, in the AQR, PQR and URX body cavity neurons. On the other hand, body size can also be regulated via multiple, non-overlapping sets of non-body cavity sensory neurons. We propose that the loss of *C. elegans* BBS function in ciliated sensory neurons leads to non-cell autonomous, aberrant cGMP-PKG signalling in body cavity neurons, which contributes to abnormal body size and delayed development.

**C. elegans** bbs mutants exhibit non-cell autonomous endophenotypes

Ciliated sensory neurons transduce environmental cues into behavioural responses. In *C. elegans* bbs mutants, defective IFT and ciliary functions are reflected by chemosensory and thermosensory deficits [13–14]. Given the restricted expression of *C. elegans* BBS...
proteins in sensory neurons, the additional bbs endophenotypes such as developmental timing, body and inferred cell size, and roaming indicate that in addition to sensory perception, sensory neurons also participate in developmental regulation in a non-cell autonomous manner. These bbs endophenotypes are not recapitulated by several dyf/IFT motor mutants, further implying that BBS proteins affect sensory neuron function in addition to their role in IFT.

While all bbs mutants share these endophenotypes, they exhibit small differences in the severity of phenotypic expression that could be attributed to specific allelic effects. Alternatively, BBS proteins could possess certain degrees of unknown functional specificity. This may not be so surprising given the difference in phenotypic expression among BBS patient populations [33–55], as well as the observation that tissue-specific BBS isoforms are responsible for some syndromic features [22,56].

The involvement of primary cilia in signalling during development [57] also positions them to affect development in a non-autonomous fashion. For example, mouse BBS proteins are required in the hypothalamus to regulate leptin receptor trafficking and to prevent the onset of obesity [31]. Ciliary dysfunction therefore contributes to increased adiposity partly in a non-cell autonomous manner. The additional phenotypes of C. elegans bbs mutants, highlights the global and non-cell autonomous consequence of sensory ciliary dysfunction, which may also account for some phenotypic features in other ciliopathy models.

The GCY-35/GCY-36 sGC regulates body size through a mechanism divergent from oxygen sensing

Previous studies established that the GCY-35/GCY-36 sGC can regulate oxygen sensation through either the body cavity neurons, or another group of neurons [40–42]. Activated by oxygen, this complex catalyzes the conversion of GTP to cGMP, which subsequently activates the cGMP-gated cation channel TAX-2/TAX-4 to initiate hypoxic avoidance responses [45]. Additional sGCs can act in body cavity neurons or other neurons under specific hypoxic conditions [45,58].

GCY-35/GCY-36 modifies body size through a mechanism partly divergent from that of hypoxic avoidance. GCY-35 is only necessary and sufficient in body cavity neurons that either have ciliated dendrites [59] or express some ciliated neuron-specific genes [60]. Furthermore, the loss of EGL-4, but not TAX-2 or TAX-4, suppresses the body size defects of bbs and dyf mutants. The loss of TAX-2 and TAX-4, in fact, slightly exacerbated bbs phenotypes (Figure S4), which may reflect an increased cGMP pool for EGL-4 activation or the loss of a potential EGL-4 phosphorylation target [35]. As well, despite some sGCs having overlapping expression profiles with GCY-35/GCY-36, other oxygen-responsive sGC mutants failed to suppress bbs-7 body size defects under standard culture conditions - possibly due to low activity under normoxia. Therefore, body cavity neurons, through GCY-35/GCY-36 activity, participate in developmental regulation through an alternate cGMP effector.

EGL-4 is present fairly ubiquitously, but the activation of EGL-4 in sensory neurons exerts a dominant influence on body size [33]. The genetic epistasis of both egl-4(gf) and egl-4(gf) alleles over that of bbs and hh3;gcy-35 argues in favour of BBS proteins and EGL-4 functioning through a shared cellular pathway to regulate body size and developmental timing. Expression of EGL-4(gf) in the body cavity neurons of gcy-35;ebb-7 mutants specifically alleviated the rescuing effect on body size, suggesting that increased EGL-4 activity, driven by increased availability of cGMP in body cavity neurons, contributes to the body size defects of some ciliary mutants (Figure 5C).

BBS proteins affect body size indirectly through body cavity neurons

The body size defects of ciliary mutants are rescued by non-overlapping sets of sensory neurons. However, restoring BBS function in body cavity neurons is insufficient to rescue the observed body size defects, giving rise to a possibility that the effect of cGMP signalling by body cavity neurons is indirectly moderated by a non-cell autonomous function of BBS proteins in ciliated sensory neurons. Furthermore, that URX, a pair of non-ciliated neurons, play a necessary role in this suppression indicates that BBS proteins are not directly influencing body size in these neurons. Our genetic analyses of the modifying effect of other GC mutants also support this scenario, as we have not found additional GC mutants that potently restore the body size of bbs mutants.

These results do not exclude the possibility that other GCs function redundantly in non-body cavity sensory neurons to influence body size through EGL-4/PKG (Figure 5D). The overexpression of egl-4(gf) in body cavity neurons was incapable of further reducing the body size of gcy-35;ebb-7 animals beyond that of bbs-7 mutants. This is in concordance with the ablation of body cavity neurons, which did not phenocopy the large body size of EGL-4 loss of function mutants, suggesting additional neuronal groups influence body size through EGL-4/PKG signalling. This study, however, establishes body cavity neurons as a predominating cGMP/PKG effector in body size regulation, and the ciliated sensory neurons as playing a key role in modulating cGMP signalling of these effector neurons.

Mechanisms on how dysfunctional ciliary sensory neurons lead to elevated cGMP/PKG signalling in these neurons are unknown. The body cavity neurons, AQR, PQR and URX do not receive extensive or direct synaptic inputs from sensory neurons where BBS proteins are sufficient to rescue body size. The non-cell autonomous effect of ciliated sensory neurons therefore suggests a potential involvement of indirect synaptic inputs, or other forms of neuronal communications, such as peptidergic and/or hormonal signalling between these neuronal groups. For example, body cavity neurons express the C. elegans homologue of the neuropeptide NPY receptor [61], making their activity susceptible to modulation by neuropeptides, some of which could be secreted by sensory neurons [62]. Sensory neurons also secrete insulin/IGF-like ligands, some of which may systematically affect neuronal
states [63–64]. Indeed, insulin and leptin have been shown to regulate the activity of specific hypothalamic neurons [63–66]. Speculatively, *C. elegans* BBS proteins could affect the secretion of multiple signals by ciliated sensory neurons to regulate cGMP/EGL-4 signalling in the body cavity neurons.

A potential involvement of aberrant cGMP signalling in ciliopathies

While aberrant PCP, Shh and Wnt signalling underlie a number of ciliopathy features, the biology behind other ciliopathy features such as photoreceptor degeneration, and reduced body size in Bbs mice [15] remains unexplained. cGMP signalling plays key roles in biological processes such as phototransduction, axonal guidance, and synaptic plasticity (reviewed in [67–68]). PKGs have also been implicated in photoreceptor degeneration and dwarfism [69–70]. It is worth exploring the involvement of cGMP signalling in the underlying pathology of BBS and other ciliopathy features.

Materials and Methods

Strains

All strains were maintained on NGM plates at 20°C. *C. elegans* *bbs*, *gcy* and *egl-4* strains were obtained from the CGC. *C. elegans* CX7102 was obtained from the Bargmann lab. Genotypes for all strains are listed in Text S1.

Mapping and cloning of *hp433*

*bbs-7(n1606);hp433* mutants were outcrossed twice against N2 by selecting animals that were genotype for *n1606* mutation, but exhibited normal body size. The *hp433* mutation was crossed into *bbs-7(ok1351)* mutants and mapped based on the suppression of small body size and roaming defects using the SNP markers in the CB4656 strain, which placed it at a 93.5 kb interval between the SNPs *pki133* and *ce11-1426*. We conclude that *hp433* encodes *gcy-35* by: 1) Injection of three overlapping fosmids covering *gcy-35*, T04D3.5, and T04D3.12, reverted the body size suppression in *hp433*; *bbs-7* animals. A fragment of *WRM641cB09* that encompassed a truncated *gcy-35*, but complete T04D3.5 and T04D3.12 failed to revert the *hp433* suppression; A genomic fragment containing only *gcy-35* fully reverted the suppression. 2) *gcy-35* animals shared the same synthetic phenotypes and genetic interactions with *bbs-7* as *hp433*, while *hp433; bbs-7(ok1351)* animals also failed to complement *gcy-35* (*ok769*);*bbs-7* (*ok1351*). 3) Sequencing of *gcy-35* identified a 2 bp deletion in exon 8.

Molecular biology, *C. elegans* phenotype examination

See Text S1.

Supporting Information

**Figure S1** *bbs* mutants exhibit a smaller body size in late larvae and adulthood stages. The body length measurements of *bbs* mutants at L4 (A) and 66-hours post-L4 (B) showed consistent size defects when compared to similarly staged wild-type animals. ANOVA with Tukey, *** p<0.001 in comparison to wild-type animals, n≥20. Data represent mean ± SD normalized against wild-type body length. (TIF)

**Figure S2** *dyf* (dye-filling) mutants show variable body size and genetic interaction with *gcy-35*(f). (A) The relative length of *dyf* mutants in comparison to wild-type animals. Some *dyf* mutants had variable degrees of body length defects, while others showed little to no change in body length. ANOVA with Tukey, *** p<0.001; ns – p≥0.05 or length difference <3.5% relative to wild-type animals, n≥30. Data represent mean ± SD normalized against wild-type body length. (B) The body size of only a subset of *dyf* mutants (dark boxes) was mildly altered by the loss of *gcy-35* (light boxes). Boxes represent 25th–75th percentile of populations with maximum and minimum values as whiskers. ANOVA with Tukey, *** p<0.001; * p<0.05; ns – p≥0.05 or length difference <3.5%, n≥30. Data represent mean ± SD normalized against wild-type body length. (TIF)

**Figure S3** *bbs* mutants show no visible defects in *gcy-35* or *gcy-36* localisation. GFP signals by *pgcy-36::GFP;gcy-35* or *pgcy-32::GFP;gcy-36* expressed in *gcy-33* (*hp433*) (A–C) or *gcy-36* (*db66*) (G–I) mutants. Strong signals were observed in the soma (orange arrowheads) and tips of the dendrites (white arrowheads) in AQR, PQR, and in the soma and dendrites of URX neurons. Expression of the same constructs in a *gcy-35* *bbs-7* (D–F) or *bbs-7* *gcy-36* (J–L) backgrounds exhibited no gross changes to localization in comparison to wild-type animals. Shown here are representative images of young adult animals. (TIF)

**Figure S4** The cGMP-gated ion channel TAX-2/4 does not suppress the body size defects of *bbs* mutants (A) *tax-2* and *tax-4* failed to rescue *bbs-7* mutant body size defects. ANOVA with Tukey, *** p<0.001; ns – p≥0.05 or length difference <3.5%, n≥20. Data represent mean ± SD normalized against wild-type body length. (B) Developmental timing in *tax-2* *bbs-7* mutants was further delayed in comparison to *bbs-7* mutant populations. ANOVA, *** p<0.001, n≥50, N≥10 replicates. Data represent mean (hours) ± SD. (C) Roaming defects of *tax-2* *bbs-7* and *tax-4* *bbs-7* animals were not different compared to *bbs-7* single animals. Kruskal-Wallis with Dunn’s, ns – p≥0.05, n≥25, N≥2 replicates. Data represent mean squares roamed ± SEM. (TIF)

**Figure S5** Mutations in other guanylate cyclases do not suppress *bbs-7* body size defects. (A) Mutant alleles of multiple sGC genes (gray bars) and rGC genes (dark bars) did not exhibit a significant modifying effect on *bbs-7* mutants. The loss of functionally redundant rGCs (*gcy-8*; *-18, -25* [52]) does not significantly modify *bbs-7* body size defects either (black bars). ANOVA with Tukey, ns – p>0.05, n≥20. (B) Suppression of *bbs-7* body size by the loss of *gcy-35* was not significantly influenced by the loss of additional rGCs. ANOVA with Tukey, *** p<0.001; ** p<0.01; ns – p≥0.05, n≥20. All data represent mean ± SD normalized against wild-type body length. (TIF)

**Table S1** A list of the *bbs* mutant alleles used in this study, outlining the type of change or deletion characterized in these strains and the resulting changes to protein translation or domains. (DOC)

**Text S1** Text S1 includes a list of strains generated and used in this study, and methods for molecular biology, phenotype analyses, statistical analyses and fluorescent microscopy. (DOC)

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Author Contributions
Conceived and designed the experiments: CAM EH MZ. Performed the experiments: CAM MPH TS MRL EH MZ. Written the paper: CAM EH MZ.

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