Title
TGFβ/BMP immune signaling affects abundance and function of C. elegans gut commensals.

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The gut microbiota contributes to host health and fitness, and imbalances in its composition are associated with pathology. However, what shapes microbiota composition is not clear, in particular the role of genetic factors. Previous work in *Caenorhabditis elegans* defined a characteristic worm gut microbiota significantly influenced by host genetics. The current work explores the role of central regulators of host immunity and stress resistance, employing qPCR and CFU counts to measure abundance of core microbiota taxa in mutants raised on synthetic communities of previously-isolated worm gut commensals. This revealed a bloom, specifically of *Enterobacter* species, in immune-compromised TGFβ/BMP mutants. Imaging of fluorescently labeled *Enterobacter* showed that TGFβ/BMP-exerted control operated primarily in the anterior gut and depended on multi-tissue contributions. *Enterobacter* commensals are common in the worm gut, contributing to infection resistance. However, disruption of TGFβ/BMP signaling turned a normally beneficial *Enterobacter* commensal to pathogenic. These results demonstrate specificity in gene-microbe interactions underlying gut microbial homeostasis and highlight the pathogenic potential of their disruption.
All animals harbor complex communities made of diverse microbes, and those of the gut are the most extensive ones. Gut microbes are often referred to as commensals—that is, causing no harm and having no benefit—and in any given condition some may indeed be just so, but overall, gut microbiota are beneficial, contributing to features as diverse as development, metabolism, immunity, fecundity, and even behaviors\(^1\). Furthermore, abnormal microbiota composition (or dysbiosis) is associated with pathology, and in some cases (i.e., obesity and potentially aging) has been shown to play causal roles\(^2\).

In determining the factors that shape microbiota composition, work in vertebrates has been instrumental in revealing a significant impact of diet\(^3\). Environmental factors, such as geography, or life style, were also shown to contribute\(^4\). Less is known about the role of genetic factors, which was suggested to have a relatively modest effect size on the microbiota\(^5\). Nevertheless, one might expect that advantages provided by beneficial microbes to a host over its peers should promote selection of genetic variants that enable colonization by such microbes, resulting in host-specific microbiotas shaped to varying degrees by genetic factors. Consistent with this, species-specific gut microbiota have been identified in various organisms, including apes, bees, termites, and Caenorhabditis elegans\(^6\). In a few instances, composition of these microbiotas was shown to be associated with host evolution. For example, in bees, the appearance of specific core gut bacterial lineages coincides with the emergence of eusocial bees from solitary ancestors\(^7\), and the composition and functional impact of microbiota was found to track phylogenetic relatedness between species of several clades, including deer mice, Drosophila, mosquitoes, and wasps\(^8\). Our own analysis of nematode microbiotas identified a significant contribution of host genetics to microbiota composition\(^8\). However, the specific genes behind such contributions are mostly unknown. Results from human studies demonstrated that host metabolism and immunity can shape the human gut microbiota. Human twin studies identified the lactase gene locus as associated with the abundance of Bifidobacterium\(^9\), and innate immune genes, such as C-type lectins, have been shown to contribute to shaping human gut microbiota function and composition\(^10\).

Whereas human studies rely on associations between natural genetic variation and microbiome composition, studies using model organisms, such as mice, can directly test the effect of a specific host function. In one such study, mice lacking CARD9, an adaptor protein required for innate immune responses, were found to harbor an altered microbiota, compromised in the production of aryl hydrocarbon receptor ligands, which led to increased susceptibility to colitis\(^11\). However, for the most part, distinguishing gene effects from inter-individual variation in vertebrate models is not trivial, hindering the ability to identify influences of host factors on microbiota composition.

An alternative is offered by invertebrate models such as Drosophila melanogaster and C. elegans. Studies in Drosophila have identified mechanisms enabling immune tolerance of gut microbes, and determining the abundance of gut commensals\(^12,13\). C. elegans offers the additional advantage of working with self-fertilizing genetically homogeneous populations, averaging-out inter-individual variation to discern gene effects. C. elegans has been used extensively for studying molecular mechanisms of innate immunity\(^14,15\), but decades of growth on monoxenic cultures, typically of an Escherichia coli strain unable to colonize healthy worms, has left a gap in the understanding of its biology and its interactions with benign microbes. This is now changing. Studies of C. elegans interactions with different food bacteria provide insights into metabolic regulation and aging\(^16\), and recent work defined a characteristic C. elegans gut microbiota, and showed that its composition was conserved across different strains and geographical locations\(^17,18,19\). Moreover, this composition bore functional significance for worms, with positive impact mainly on development and on immunity, provided typically by Pseudomonadaceae and Enterobacteriaceae bacteria, including host-specific contributions (reviewed in\(^20\)).

Taking advantage of the availability of C. elegans mutants, we examined the contribution of host genes to shaping the C. elegans gut microbiota. RNAseq identified genes involved in digestion and in innate immunity as those upregulated during interactions with complex microbiotas. Analysis of mutants for genes central to these processes, using synthetic communities composed of previously isolated worm gut commensals, and providing a defined environment, identified a role for Transforming Growth Factor (TGF)β/Bone Morphogenetic Protein (BMP) signaling in controlling bacterial abundance of Enterobacter commensals and in determining their contributions to the host.

**Results**

**Genes modulated during interactions with complex microbiota.** RNAseq analysis was performed to identify C. elegans genes and processes involved in host–microbiota interactions, comparing gene expression in worms grown on complex environmental microbiotas to that in worms grown on E. coli. Two comparisons were performed: one in composted soil microcosms (autoclaved compost reconstituted either with the microbiota from an unautoclaved batch of the same soil, or with a saturated E. coli culture); the second on plates, seeded either with E. coli or with synthetic microbiotas prepared with equal portions of 30 C. elegans gut isolates representing the main core microbiota families (SC1, see Methods). Analyses were performed in age-matched adult worms from synchronized populations; three independent populations were analyzed per group. Measurements were obtained for 28,555 unique RNA transcripts (measured in at least one sample), representing 18,873 genes (see Data availability).

In worms raised on the synthetic community, 127 genes were significantly upregulated and 163 genes were significantly downregulated compared with worms raised on E. coli (false-discovery-rate-corrected q-value < 0.05, likelihood ratio test, Supplementary Data file 1). Enrichment was found among the upregulated genes for immune genes, as well as for hydrolases (peptidases in particular) (Fig. 1a, Supplementary Data file 2). This is in spite of lack of any indication that SC1 included pathogens that compromised worm survival (Supplementary Fig. 1a), suggesting that elevated immune activity underlies normal host–microbiota interactions. Among the downregulated genes, only broad gene ontology (GO) terms, such as catabolic processes, were enriched.

Many more genes were affected in worms raised in composted soil microcosms: 1269 genes were significantly upregulated and 1815 downregulated, compared with worms grown on E. coli–supplemented soil (Supplementary Data file 1). The larger number of differentially expressed genes in soil-grown worms compared with those raised on SC1 indicates that C. elegans does not respond to complex microbiotas in a stereotypical way and suggests that the extent of changes in gene expression may depend on microbial diversity. Among both upregulated and downregulated genes, we found enrichment for genes associated with developmental programs, and to a lesser degree (and specific for downregulated genes) with reproduction (Supplementary Data file 2). In agreement with this, gravid worms harvested from normal soil microcosms held fewer eggs in their uterus compared with those raised on E. coli, either on plates or in soil (one row of eggs versus two). Using SC1 plates, we followed worm development and reproduction more closely. Worms raised on
SC1 started laying eggs 1–2 h earlier than worms raised on E. coli, but laid in total 25% fewer eggs (Supplementary Fig. 1b). This correlated with the identified expression trends, and suggested that exposure to a diverse microbiota modulated host development resulting in a trade-off between development and fecundity. Another prominent trend emerging in microcosm-raised worms was enrichment for upregulated genes involved in response to external stimuli, many of which were immune genes (Supplementary Table 2). This corroborated the trends observed in worms raised on SC1.

The overlap between genes significantly upregulated on microcosm microbiotas or on SC1 included 25 genes (Table 1). The great majority of these (21/25) are reported to be expressed in the intestine (Wormbase [http://www.wormbase.org]). These included C-type lectins, saposins, peptidases, as well as enzymes involved in sphingomyelin metabolism, and were significantly enriched for genes associated with defense and immune responses (p = 6E–5, Bonferroni corrected) and for genes encoding hydrolytic enzymes (p = 4E–5), including several lysosomal enzymes (p = 2E–6), pointing to immune functions as a common denominator among host factors that interact with the microbiota. Expression patterns for several of the genes were consistent across experiments, pointing to immune functions as a common denominator among host factors that interact with the microbiota. Expression patterns for several of the genes were consistent across experiments, pointing to immune functions as a common denominator among host factors that interact with the microbiota.

Among the genes upregulated by the exposure to diverse microbiotas were genes previously described to respond to environmental bacteria, specifically to a Comamonas isolate33 (11/25 genes, Supplementary Table 1). Although this is not surprising considering that SC1 included a Comamonas isolate, upregulation of these genes in worms raised in compost microcosms suggests similar interactions in natural-like conditions.
environments, which is in agreement with previous reports of *Comamonas* species being part of the *C. elegans* gut microbiota. Further in agreement with enrichment for immune genes, a significant overlap was observed between the microbiota-upregulated 25 genes and targets of central immune regulators, including the p38 pathway, the DAF-16/FOXO transcription factor, and TGF-β signaling is involved in shaping the gut microbiota, and TGF-β (kdm25) mutants, which are involved in feeding behavior, which might also affect microbiota structure. All worm strains were raised on plates with the SC1 synthetic diet, which might also affect microbiota structure.

| Gene     | SC1/E. coli | q-Val | Soil/E. coli | q-Val | Concise description |
|----------|-------------|-------|--------------|-------|---------------------|
| Y65B4R1  | 48.4        | 1.82  | 6.4          | 9.3E-24 | Defense response; predicted hydrolase |
| F53A9.8  | 11.3        | 2.4E-14 | 5.2         | 1.4E-08 | Defense response to G + bacteria; intestinal |
| clec-52c | 13.5        | 1.2E-11 | 12.7       | 1.2E-25 | A Collectin homolog; defense response to G + bacteria; expressed in pharynx, neurons, intestine |
| clec-14  | 7.9         | 4.4E-09 | 2.0         | 2.1E-03 | Encodes an aspartyl protease |
| asah-1c  | 1.8         | 1.3E-05 | 4.3         | 4.0E-39 | N-acylphosphosine amidohydrolase 1 ortholog; determination of adult lifespan; intestinal |
| clec-65c | 3.4         | 1.4E-05 | 1.3         | 1.4E-02 | Epoxide hydrolase 1 ortholog; immune response |
| ssp-8b   | 1.9         | 3.3E-05 | 8.8         | 6.0E-44 | Porosapin-like 1 ortholog; enriched in: intestine, germ line, PVD/OLL neurons |
| sci-2c   | 1.8         | 2.6E-04 | 4.1         | 9.1E-21 | A SCP/TAPS-domain protein. Expressed in the intestine |
| psp-1b   | 2.6         | 3.2E-04 | 4.0         | 1.3E-15 | Innate immune response |
| hpo-15c  | 9.1         | 7.8E-04 | 3.6         | 2.7E-09 | Polyamine oxidase (exo-N4-amino) oxidase; determination of adult lifespan |
| C14C6.2c | 2.2         | 2.0E-03 | 2.2         | 4.8E-02 | Enriched in: intestine, PVD/OLL neurons |
| arg-3c   | 13.3        | 2.0E-03 | 3.6         | 3.6E-04 | Innate immune response |
| clec-84c | 2.0         | 2.4E-03 | 2.0         | 4.8E-03 | Enriched in: intestine, pharyngeal muscle, germ line |
| lips-15c | 3.2         | 4.3E-03 | 5.3         | 4.7E-04 | Predicted hydrolase |
| cyp-37B1c| 4.3         | 4.4E-03 | 2.6         | 4.0E-02 | Cyt P450 homolog; defense response to G + ; intestinal |
| cpr-3c   | 4.6         | 1.0E-02 | 8.7         | 3.9E-12 | Cathepsin B ortholog; involved in embryo development, immune response; expressed: pharynx, pharyngeal-intestinal valve, intestine, rectal gland |
| nuc-1c   | 2.0         | 1.3E-02 | 2.3         | 3.1E-07 | A DNase II homolog; required for DNA degradation during apoptosis, and degradation of dietary DNA |
| asm-3    | 14.1        | 2.3E-02 | 23.8        | 8.1E-40 | Sphingomyelin phosphodiesterase 1 ortholog; determination of adult lifespan |
| asp-1c   | 2.3         | 2.7E-02 | 2.8         | 9.9E-09 | Cathepsin D homolog (aspartic protease); transcribed in late embryo/early larvae intestine; not observed later |
| col-77c  | 9.8         | 2.9E-02 | 32.0        | 6.5E-08 | Encodes a cuticular collagen |
| C53B7.3c | 2.0         | 3.2E-02 | 4.0         | 1.5E-10 | Enriched in: germ line, intestine, PVD/OLL neurons |
| cpl-2c   | 1.8         | 3.6E-02 | 2.3         | 2.9E-13 | Encodes an aspartyl protease homolog, required for degenerative (necrotic-like) cell death in neurons |
| ZK896.5c | High        | 3.9E-02 | 5.1         | 8.1E-03 | Epoxide hydrolase 1 ortholog; enriched in: intestine, pharyngeal muscle |

*Fold change in worms raised on complex microbiotas compared with worms raised on E. coli.*

*Known expression in the intestine.*

*When expression in E. coli was undetected (0).*

TGF-β/BMP signaling is involved in shaping the gut microbiota. Since immunity emerged as a common denominator in worm responses to complex microbiota, immune and regulators as potential players in shaping these responses, we tested the significance of disrupting such regulators for the composition of the worm gut microbiota. Mutants examined included *pmk-1* (kdm25) and *tir-1(qd4)*, disrupted for the p38 MAPK ortholog and its SARM adaptor, respectively, *dbl-1* (nk3) mutants, lacking the TGF-β/BMP-like ligand DBL-1; *daf-16(mu86) mutants, disrupted for a FOXC transcription factor central for stress resistance, immunity, and longevity, along with mutants for one of its targets, the *ctl-2 catalase*; and *tol-19(n233)* mutants, disrupted for the sole toll receptor homolog in *C. elegans*. As a control, we examined *tnt-3(ok1011) mutants, which are defective in grading ingested bacteria, allowing more intact bacteria into the intestine. Beyond factors that operate in the intestinal niche, we examined mutants for *osm-6* and *ttx-3*, which are involved in feeding behavior, which might also affect microbiota structure. All worm strains were raised on plates with the SC1 synthetic community, and their gut microbiota size (total bacterial load) and composition were evaluated in the first day of adulthood, using qPCR with eubacterial and taxa-specific primers, calibrated to known quantities of a full-length ampiclon of the 16S ribosomal RNA gene (rDNA) from the respective taxa (see Methods). Measured taxa included *Enterobacteriaceae* and *Pseudomonadaceae*, which are the most abundant families in the *C. elegans* gut, as well as the less common *Bacillaceae*, which are nevertheless part of the worm core gut microbiota.

The *tnt-3* control strain showed a significantly greater gut bacterial load than wild-type animals (Fig. 1b). This was expected, as it is impaired in the grinding of bacteria. In contrast, most tested mutants showed only small or insignificant changes in their total bacterial load. The exception was *dbl-1* mutants, which demonstrated a threefold increase. This was associated with increases in the abundance of *Enterobacteriaceae*, and more variably, of *Pseudomonadaceae* (Figs. 1c, d). The abundance of *Bacillaceae* family members in wild-type or mutant animals was variable to the extent that no clear differences could be discerned (Fig. 1e).

Although total bacterial load in *tnt-3* mutants was much larger than in wild-type animals, microbiota composition, represented by the relative abundance of measured taxa, did not significantly differ from the wild type. This further suggests that *tnt-3* mutants have an intact immune system and that the loss of *tnt-3* specifically affects bacterial load and composition in the gut microbiota.
change, indicating proportional increases in different microbiota members (Fig. 1f). A similar trend in taxa relative abundance was observed in feeding behavior mutants, suggesting that at least in the context of the plate microbiota, food preference was not a significant factor shaping the gut microbiota. In contrast, mutants for stress and immunity regulators, which had the same total bacterial load as wild-type animals (excluding dbl-1 mutants), showed significantly altered composition: pmk-1, tir-1, and daf-16 mutants showed relative expansion in taxa (one or more) for which we did not have specific primers, i.e., Comamonas, Aeromonas, or others (bundled as “other”), on the expense of the taxa that in wild-type animals are the dominant ones, Enterobacteriaceae and Pseudomonas (Fig. 1f). dbl-1 mutants, on the other hand, harboring an expanded gut microbiota, showed a significant increase in the relative abundance of Enterobacteriaceae. Both the increase in total bacterial load in dbl-1 mutants, and the particular expansion of Enterobacteriaceae were confirmed with colony-forming unit (CFU) counts, evaluating the number of live bacteria in the intestine of wild-type and dbl-1 animals (Fig. 1g).

**BMP/DBL-1 signaling specifically affects Enterobacter abundance.** Focusing on the prominent effects of dbl-1 disruption, we examined whether microbiota expansion in dbl-1 mutants was a general trend in these mutants, or unique to the particular composition of SC1. To this end, we raised wild-type worms and dbl-1 mutants on a different synthetic community (SC2), in which approximately two-thirds of SC1 strains were replaced with distinct isolates, while keeping the same genera represented (Supplementary Table 2). Both on SC1 and on SC2, dbl-1 mutants showed a significantly greater bacterial load compared with wild-type worms ($p < 0.001$, Fig. 2a), as well as a threefold more Enterobacteriaceae (Fig. 2b). Furthermore, when grown in composted soil microcosms, providing natural-like microbial diversity, dbl-1 mutants showed only a modest increase in total bacterial load, but a significant twofold increase in the abundance of Enterobacteriaceae (Fig. 2c–f). Together, these observations support the notion that gut microbiota expansion in dbl-1 mutants, and in particular an increase in the abundance of Enterobacteriaceae, represented a general feature of these mutants.

To determine whether any specificity could be discerned in the effects of dbl-1 disruption on the gut microbiota, we created modified versions of SC1 with reduced diversity. The first modified community configuration, SC1R, excluded all Pseudomonas isolates, as well as most of the Enterobacteriaceae (Supplementary Table 2). While dbl-1 mutants raised on SC1 have an expanded microbiota compared with wild-type animals, those raised on SC1R did not show this expansion (Fig. 3a), indicating that disruption of dbl-1 had somewhat restricted effects, allowing a bloom of Pseudomonas and/or Enterobacteriaceae, but not of other members of the community. In contrast, tnt-3 mutants raised on SC1R demonstrated an expanded microbiota similar to when raised on SC1, supporting the indiscriminate effects of defective grinding on microbiota expansion. A subter modification of SC1, SC1R*, which excluded only Enterobacteriaceae species (not all), also abolished the increase in microbiota size in dbl-1 mutants, further indicating specificity toward certain Enterobacteriaceae species. Finally, eliminating only Enterobacter isolates from the synthetic community (SC1**), leaving-in several other Enterobacteriaceae species (e.g., Escherichia sp., Buttiauxella sp.), was sufficient to abolish microbiota expansion in dbl-1 mutants, pointing at
**Enterobacter** isolates as those affected by *dbl-1* disruption. Supporting this, re-examination of the microbiota in *dbl-1* mutants raised on SC1, using primers specific for the *Enterobacter* *hs60* gene, revealed an increase in the *Enterobacter* load in *dbl-1* mutants that could account for the entire increase in bacterial load observed in these animals (Fig. 3b, as compared with Fig. 3a).

The observed increase in *Enterobacter* abundance in *dbl-1* mutants could be due to direct effects of host TGFβ/BMP signaling on *Enterobacter* colonization and/or proliferation, or caused indirectly by changes in microbial interactions affecting the balance between *Enterobacter* and its competitors. To examine which of the two alternatives is more likely, we raised worms on a tdTomato-expressing derivative of one of the SC1 isolates, a previously characterized *Enterobacter cloacae* commensal of *C. elegans*, CEN2ent1 (shortened here to CEent1) [18]. Fluorescent microscopy of adult *dbl-1* and wild-type animals demonstrated a significant increase in the abundance of these bacteria in *dbl-1* mutants compared with wild-type animals (Fig. 3c). As no other bacteria are present to affect CEent1 abundance, these results indicate that *dbl-1* disruption directly affected *Enterobacter* abundance.

**Enterobacter bloom is not due to mutants’ impaired development.** TGFβ/BMP signaling involves ligand binding and activation of heterodimer receptors, downstream SMAD transcriptional regulators (Sma and Mothers against decapentaplegic homologs), and co-activators [43]. In *C. elegans*, this pathway contributes to immunity, but more visibly to development, and all known mutants have small body size. Whereas the smaller *dbl-1* mutants showed a greater bacterial load than wild-type animals, we nevertheless wished to ascertain that this bloom was not caused (perhaps indirectly) by altered development. Disruption of the TGFβ type 1 receptor gene, *sma-6*, or the R-SMAD (Receptor-regulated SMAD) gene, *sma-3*, both led to an *Enterobacteriaceae* expansion, either slightly smaller than in *dbl-1* mutants (*sma-6*) or larger (*sma-3*) (Fig. 4a–c). In contrast, mutants for the *sma-9* co-activator, which are as small as *dbl-1* mutants [43], showed no bacterial expansion whatsoever (Fig. 4a, b). Fluorescent imaging, using the tdTomato-expressing CEent1 to follow *Enterobacter* colonization, corroborated these results (Fig. 4d, e), further diminishing the likelihood that *Enterobacter* bloom in TGFβ/BMP mutants was due to altered development in mutants. This in turn leaves impaired immunity as the more likely cause for the *Enterobacter* bloom. In line with this, while *sma-6*, *sma-3*, and *dbl-1* are central immune regulators, *sma-9* was reported to be redundant for immune gene induction [43].

**TGFβ/BMP-exerted control acts primarily in the anterior gut.** To examine the opposite case of *dbl-1* disruption, we employed a strain overexpressing *dbl-1* from an integrated genomic fragment (*dbl-1 o/e*) [39]. As observed in animals raised to adulthood on *tdTomato-CEent1*, *Enterobacter* load in *dbl-1 o/e* animals was overall comparable to that in wild-type animals (Fig. 5a, b). However, the distribution of bacteria in the gut was very different: although wild-type animals showed prominent accumulation of *Enterobacter* in the anterior gut, this was mostly missing in *dbl-1 o/e* animals (Fig. 5a, b). This suggested that TGFβ/BMP signaling exerted its control over *Enterobacter* colonization/proliferation mainly in the anterior gut.

*DBL-1* is expressed primarily in neurons, but its receptors and downstream mediators are expressed in the epidermis, pharynx and in the intestine [43]. To gain insight into where TGFβ/BMP signaling might function to delimit *Enterobacter* accumulation, we took advantage of *sma-3*(**30**) mutants, which are heavily colonized with *Enterobacter*, and a panel of transgenic strains employing tissue-specific promoters to rescue *sma-3* expression in different tissues [46]. Expression of *sma-3* from its endogenous promoter effectively restored accumulation of *tdTomato-CEent1* to wild-type levels (Fig. 5c). Expression of *sma-3* from the epidermal *dpy-7* promoter also delimited *Enterobacter* accumulation, although not quite to wild-type levels, and expression from the pharyngeal *myo-2* promoter showed *Enterobacter* accumulation to levels intermediate between those of wild-type and those in *sma-3* mutants. In contrast, worms with intestinal *sma-3* expression (relying on the *vha-6* promoter) showed *Enterobacter* accumulation that was not significantly different from that seen in *sma-3* mutants. Similar trends were observed in measurements performed on the anterior gut or on its posterior parts (Supplementary Fig. 3). These results suggest multi-tissue contributions of TGFβ/BMP signaling to controlling intestinal *Enterobacter*, with the epidermis providing the more dominant input, whereas local TGFβ/BMP signaling in the intestine appearing to be mostly redundant.

**TGFβ/BMP disruption turns an *Enterobacter* commensal to pathogenic.** CEent1 was previously shown to be beneficial for *C. elegans*, accelerating development compared with worms raised on the standard *E. coli* food, and enhancing resistance to the pathogen *Enterococcus faecalis* [18] (Fig. 6a). A 4-h exposure of worms to the commensal late in development (at the L4 stage) was sufficient to increase resistance to subsequent infection (Supplementary Table 3). However, *dbl-1* mutants developing on
Fig. 4 Disruption of TGFβ/BMP signaling alters gut microbiota size and composition independent of effects on body size. **a**, **b** Bacterial load in wild-type worms and TGFβ mutants—dbl-1(nk3), sma-6(wk7), sma-3(e491), and sma-9(wk55), raised on the SC1 community, showing abundance of all Eubacteria (**a**), or Enterobacteriaceae (**b**). Average ± SD of two independent experiments. Measurements were performed on a pool of n = 30 worms per experiment. **c** Relative abundance of all measured taxa (including also Pseudomonadaceae and Bacillaceae), calculated based on the absolute quantities, as in Fig. 1; light gray bar represents relative abundance of all ‘other’ bacterial groups not directly measured. *p < 0.05 (analysis of variance (ANOVA)) compared with wild-type animals; N(wt) = 47, N(dbl-1) = 42, N(sma-3) = 66, N(sma-9) = 89. Size bar, 0.1 mm

**Fig. 5** TGFβ/BMP signaling controls Enterobacter colonization in the anterior intestine through multi-tissue contributions. **a** Representative images of wild-type and dbl-1 overexpressing (o/e) worms raised to adulthood on tdTomato-expressing CEnt1. Asterisks mark the posterior pharynx, size bar, 0.1 mm. **b** Box plots (described under Fig. 4) quantifying fluorescent signal (normalized to area) in images as in **a**, either in the anterior gut (dashed line in **a**), or posterior to that. n = 38 (wt), 50 (dbl-1), 28 (dbl-1 o/e); **p < 0.0001, *p < 0.05**, generalized linear model. **c** Similar quantification in the anterior gut of wild-type worms, or sma-3 mutants, as well as in sma-3 derivatives with transgenic tissue-specific sma-3 expression. Statistically distinct groups (p < 0.05) are marked with different letters (analysis of variance (ANOVA) followed by a Tukey’s post hoc test; n = 71(wt), 60 (sma-3 mutants), 48 (endogenous sma-3 promoter), 86 (epidermal sma-3), 40 (pharyngeal sma-3), 33 (intestinal sma-3), all hermaphrodites)

CEent1 (or exposed to it late in development) no longer showed enhanced pathogen resistance and instead were significantly more susceptible (Fig. 6b and Supplementary Table 3). sma-3 mutants showed an even greater susceptibility (Fig. 6c, d). Furthermore, sma-3 mutants raised on tdTomato-CEent1 and shifted late in development to pathogen were still colonized 24 h after the shift (9 out of 24 examined) (Fig. 6f), and even 48 h after the shift, at which point colonization was further observed in three out of five
cadavers. In contrast, wild-type animals showed no CEent1 colonization persisting after the shift. These results indicate that impaired TGFβ/BMP signaling results in a more persistent Enterobacter colonization, turning the normally beneficial commensal to pathogenic. Interestingly, dbl-1 overexpressing worms, which were more resistant to E. faecalis to begin with, showed no significant increase in their resistance when initially exposed to CEent1 instead of E. coli (Fig. 6e).

Discussion
With the microbiome linked to many aspects of host health, understanding the interactions between host and its microbes is imperative for the development of therapeutic strategies aiming to alter the microbiota. An important aspect of this is understanding how host genes determine abundance and function of gut commensals. This understanding has been slow to emerge, to some extent due to inter-individual variation in microbiota composition in vertebrate models, which masks gene effects. Here, we demonstrate the utility of C. elegans as a model organism facilitating the identification and characterization of such gene effects, including the distinction between effects on microbiota size (total microbial load) and microbiota composition. Using this model, and screening candidate regulators, we identified a role for TGFβ/BMP immune signaling in controlling the abundance of members of the genus Enterobacter, common inhabitants of the worm gut, which affect host development and immunity. TGFβ/BMP-exerted control was focused on restricting Enterobacter accumulation in the anterior gut, but full effects depended on contributions from several tissues. Disruption of TGFβ/BMP signaling resulted in an Enterobacter bloom, and turned an otherwise useful commensal to pathogenic. Gut dysbiosis is typically considered a condition that enables invasion and proliferation of environmental opportunistic pathogens. Our results demonstrate that given impairment in host immunity and ensuing dysbiosis, pathogenicity can emerge directly from otherwise beneficial members of the gut community.

Current understanding of how host genes and processes shape microbiota composition highlights the importance of mucosal structure and immunity. Genes identified as affecting abundance of gut bacteria include antimicrobial peptides, pattern recognition receptors, cytokines, and genes affecting IgA antibody production, as well as enzymes glycosylating barrier mucins, which provide sugars harvested by gut bacteria. Our RNAseq analysis indicated a similar trend in C. elegans, as worms interacting with a diverse microbiota had elevated expression of genes, mostly intestinal, which are associated with host immunity. Some of these genes encode hydrolytic enzymes, which may serve immune functions, but could also be relevant for releasing nutrients from susceptible ingested bacteria for the benefit of resistant gut commensals. Mutant analysis supported the importance of host immunity in shaping the gut microbiota. Since many of the identified overexpressed genes are members of multi-gene families, implying potential functional redundancy, we focused instead on central upstream immune regulators. The contribution of immune regulators to microbiota composition was conspicuous, and in the case of dbl-1 mutants was recapitulated with different synthetic communities, as well as (with regards to the main feature of Enterobacteriaceae expansion) in worms raised in a natural-like compost microcosm. In contrast, disruption of daf-16, which encodes a general stress resistance regulator and contributes also to immunity, had no effect on microbiota composition. Feeding behavior may also be thought to affect microbiota composition. However, disruption of osm-6 and ttx-3, did not show any effect. This cannot completely rule out involvement of food sensing and feeding behavior in determining microbiota composition, as the synthetic microbiota used is solely made of gut isolates (i.e., commensals) and is thoroughly mixed; under more natural conditions regulators of feeding behavior may contribute by identifying pockets of desirable bacteria in a non-homogeneous environment. Nevertheless, the finding that impaired grinding in tnt-3 mutants affected microbiota size but not composition supports an important role for post-feeding mechanisms, likely those defining the intestinal niche, in shaping microbiota composition.

TGFβ/BMP signaling regulates diverse processes in C. elegans, including growth, male tail development, and immunity. Our results point at its roles in immune regulation as those affecting gut bacterial load. To date, not much is known about how TGFβ/
BMP signaling regulates intestinal immunity33,34. Our results indicate that in controlling commensal colonization it relied on multi-tissue contributions, primarily from the epidermis, but also from the pharynx. Contributions of TGFβ/BMP signaling in the intestinal niche itself were marginal at best (although it seemed to affect Enterobacter accumulation in the posterior gut more than it did in the anterior, see Supplementary Fig. 3). This suggests that TGFβ/BMP signaling may affect the Enterobacter niche indirectly, potentially involving an additional signaling pathway that targets the gut. This mode of regulation may also apply to TGFβ/BMP involvement in resistance to intestinal pathogens. At the same time, results from experiments with dbi-1/o worms suggest that the Enterobacter niche in question is the anterior gut, as over-expression of the ligand and the presumed over-activation of the pathway prevented Enterobacter accumulation particularly in that region. It is further possible that colonization of this region is essential for the protective effects of Enterobacter, explaining the inability of C. elegans to enhance infection resistance in dbi-1/o animals.

The role of immunity in shaping the gut microbiota may be thought to be nonspecific, especially if considering the reliance of C. elegans solely on innate immunity (as all invertebrates). If so, disruption of any immune pathways will cause a relatively indiscriminate proliferation of gut commensals. However, this was not the case, as bacterial proliferation was selective and specific to the disrupted pathway: disruption of TGFβ/BMP signaling led to blooming only of Enterobacter species, not affecting other members of the microbiota, including other isolates of the Enterobacteriaceae family; and disruption of p38 signaling did not affect the abundance of major examined taxa (Pseudomonadaceae and Enterobacteriaceae), and only caused an increase in relative abundance of other taxa, yet to be defined. It might be speculated that such specificity depends on the profile of immune effectors regulated by each of the pathways, and differential susceptibility of different gut microbes to components of these profiles.

TGFβ signaling is highly conserved, regulating development and immunity also in vertebrates35. Whereas TGFβ/BMP signaling was shown to be associated with immune responses both in Drosophila and in vertebrates36,37, it is the role of TGFβ signaling in vertebrate T-cell differentiation and mucosal homeostasis that is better known38. Of particular relevance is the role of TGFβ signaling in production of IgA antibodies, which delimit microbiota proliferation in the gut39. Early results showed that TGFβ1 deficiency correlated with ulcerative colitis-associated colon cancer, and studies in mice suggested that colitis and cancer might depend on gut microbes40. More recently, it has been shown that deficiency in TGFβ signaling in intestinal dendritic cells led to changes in epithelial structure, Enterobacteriaceae-driven dysbiosis, and colitis40. Together, these reports describe a central role for TGFβ signaling in controlling gut microbial proliferation. Our results in C. elegans suggest that this role is conserved, even without specialized immune cells or antibodies, and further suggest particular importance, with ancient origins, for TGFβ signaling in controlling gut commensals of the Enterobacteriaceae family.

Methods
C. elegans strains and maintenance. Strains used in this study included N2 wild-type worms, the mutant strains pmk-1(ok252), tir-1(qdk4), tol-1(n2003), dbi-1(ok3), sma-3(ok119), sma-6(ok72), sma-9(e553), ada-16(m986), cl-2(ok1137), ced-6(p111), ttx-3(ok122), and tnt-3(ok1011), and the dbi-1 overexpressing strain BW1940 (which harbors several copies of the genomic dbi-1 locus), all acquired from the Caenorhabditis Genetic Center. In addition, strains with tissue-specific expression, gratefully received from Meng Wang’s lab, included sma-3(wk30)him-5(e1490), qh-2[msp-3::GFP];sma-3+;rol-6], sma-3(wk30)him-5(e1490);psmy-2p::GFP;psma-3+;rol-6], sma-3(wk30)him-5(e1490);psmy-2p::GFP;psma-3+;rol-6], sma-3(wk30)him-5(e1490);psmy-2p::GFP;psma-3+;rol-6], and sma-3(wk30)him-5(e1490);psmy-2p::GFP;psma-3+;rol-6], sma-3

(wk30);him-5(e1490) were obtained by picking non-rollers, who lost the extra-chromosomal transgene. All strains were cultured on nematode growth media (NGM) at 25 °C.

Worm growth in soil and harvesting. Fresh local soil was supplemented with chopped over-ripe apples. The soil produce mixture was allowed to decompose for 2 weeks over the lab, cleared of garden variety worms by autoclaving, and re-inoculated with a microbial extract from the original batch of unautoclaved soil. Approximately 180 worms (1800 rpm from a worm suspension (1800 rpm) from re-suspended and vortexed in M9 salt solution)13. Initially germ-free L1 larvae, obtained by bleaching of gravid worms to release eggs, and hatching them on NGM plates without food, were transferred to soil and grown at 25 °C for 3 days. One batch of prepared soil was split into separate 20 mL glass beakers (5 g per vial), and independent worm populations were raised in each (four replicates per genotype). Approximately 100–200 gravid worms were harvested from each population using a Baermann funnel lined with two layers of tissue paper, washed extensively (6×), and surface sterilized on plates containing 100 μg/ml gentamicin prior to DNA extraction, using the MO BIO PowerSoil DNA isolation kit (#12888)13.

Synthetic community and worm growth. Bacteria were previously isolated from worms grown in soil, and identified by 16S sequencing or multi-locus sequencing13,18,37. Enterobacteriaceae isolates were cultured on an Enterobacteriaceae-selective medium (Violet Red Bile Glucose, VRBG), whereas all others were cultured on Lysogeny Broth (LB) agar plates. A total of 30 isolates were selected for the synthetic community, representing most core taxa of the previously characterized C. elegans gut microbiota taxa13 (Supplementary Table 2). Several configurations of this community were used, including SC1 and SC2, which include the same genera, but differ in the specific strains used; SC1R resembles SC1, but lacks Enterobacteriaceae and Pseudomonas isolates; SC1R* lacks most of the Enterobacteriaceae isolates; and SC1R** contains all the SC1 isolates, except for the Enterobacter isolates. For each microbiota, isolates were grown on soil at 37 °C overnight with shaking. Saturated cultures were combined in equal proportions, concentrated 10×, and seeded onto NGM plates approximately 30 min before the addition of eggs, obtained from bleaching gravid worms.

For each worm strain, 2–3 independent populations of worms, synchronized at L1, were grown on the synthetic microbiotas at 25 °C for 3 days. Adult worms were washed, surface sterilized, and used for DNA extraction, as described for worms raised on soil.

DNA extraction. For each plate, 30 washed worms were transferred to a lysis buffer of a constant volume (per 30 worms: 6 μL of 10X PCR buffer (Invitrogen #1104011), 3.6 μL of Proteinase K (Fisher Scientific #EO0491), and 50.4 μL of PCR-grade water), and were lysed at 60 °C for 1 h, followed by a 15-min incubation at 95 °C to inactivate the proteinase K. Samples were stored at −20 °C until use. To rule out measurement biases introduced by DNA extraction method, DNA was extracted from synthetic communities mixed in different ratios either using the proteinase K protocol, or the MO BIO PowerSoil DNA isolation kit (#12888).

RNAseq library preparation and analysis. Synchronized worm populations, initiated with eggs obtained from bleached gravid worms, were grown on either NGM plates seeded with E. coli, NGM plates seeded with the synthetic community, in autoclaved soil supplemented with E. coli, or in composted soil, as described above (three independent populations per condition). RNA was extracted from gravid worms using a modified CTAB protocol with Aluminum Ammonium Sulfate and PEG precipitation62, followed by purification with the Qiagen AllPrep kit (#802004) to separate RNA from DNA. Sequencing libraries were prepared from total RNA using the Illumina TruSeq RNA Library Kit v2 (RS-122-2001), which synthesizes complementary DNA (cDNA) from mRNA fragments using random primers, and provides 24 indexed adapters for multiplex sequencing. Paired-end sequencing was performed on Illumina HiSeq4000, generating 100 base-pair reads, from which adapter sequences, as well as low-quality reads, were removed prior to analysis, providing 30,270,407 reads/sample on average. Both forward and reverse sequences were used for analysis, employing Kallisto to quantify transcript abundance, and Sleuth to identify differentially expressed genes, using a false discovery rate-corrected p-value (q-value) cutoff of 0.05, as previously described63. GoTermFinder was used to identify enrichment among identified genes for regulators of annotated processes or functions. Enrichment for gene targets of various regulators was calculated using the hypergeometric test.

RNA extraction and qRT-PCR. RNA was extracted from 100 to 200 worms per group using Trizol (Invitrogen). Genomic DNA contamination was removed with Turbo DNase (QIAGEN), cDNA was synthesized using iScriptTM (Bio-Rad), and quantitative real-time PCR was carried out using Bio-Rad’s iQAdvanced Universal SYBR Green Supermix, and on a StepOnePlus system (Applied Bio). Ct values were normalized to the respective actin values for each sample, and are presented as fold change over wild-type worm expression value. See Supplementary Table 4 for primer sequences.
Quantifying bacterial load (microbiota size) and microbe abundance. Quantitative PCR (qPCR) was used to estimate taxa abundance by quantifying their respective DNAs (in parallel to 16S rDNA), with host-specific primers 806F and 895R (Supplementary Table 4), and taxon-specific primers were used to quantify Enterobacteriaceae 16S (Ent_MB_F and R), Pseudomonadaceae 16S (Pse435F, Pse686R), and Bacillus 16S (BacillF, BacillR) in DNA extracts from worm samples obtained either using standard proteinase K based lysis, or PoweRSoil, as described above. In addition, taxon-specific primers targeting the Hsp60 gene were used to quantify Enterobacter cloacae (Ent-Hsp60F, Ent-Hsp60R). Cycling parameters: 95 °C for 5 min; 45 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 15 s [30 s for Bacilli]; 72 °C for 5 min. Specificity of each primer pair used was confirmed using DNA extracted from individual members of the synthetic communities. This further ensuring no amplification of C. elegans mitochondrial DNA, which can be amplified with some primers. All measurements were performed in duplicate with 1 μl of DNA extract as template, prepared from 30 worms in 60 μl of lysis buffer (for worms grown on synthetic microbiota) or from 100 worms in 30 μl (DNA extraction from worms grown on soil), to enable comparison of values between worm populations. DNA quantities were estimated using standard curves created for each taxa from qPCR measurements of 10-fold serial dilutions of an appropriate full-length 16S bacterial rDNA in known concentrations, ranging from 25 ng to 0.2 pg/μl (amplified using the same primers as described above); E. coli 16S was used for calibrating both eubacterial and microbiota 16S (BacilliF, BacilliR) in DNA extracts from worms grown on soil), to enable comparison of values to early time points, representing the bulk of the population.

CFU counts. Following harvesting and washing, worms were ground (10 worms in 250 μl of M9), releasing live bacteria, which were serially diluted. Aliquots were plated onto either LB or VRBG plates, which were incubated at 37 °C overnight before counting.

Fluorescently tagged Enterobacter cloacae. A plasmid that constitutively expresses tdTomato from the Enterobacter cySb promoter (fragment 132a)64, with kanamycin resistance, was transformed into C. elegans strain V58366. Dead worms were counted to assess infection resistance. All imaging experiments were performed using a one-way analysis of variance (ANOVA) for repeated measures, to account for within-experiment replication (line function from the nlm R package), with host genotype as a fixed effect, and within-experiment replication as a random effect67. Differences in worm colonization by C. elegans::GFP were evaluated using ANOVA (sow function in R) followed by Tukey’s Honest Significant Difference (HSD) post hoc test when all comparisons were relevant, or linear model when one condition could serve as a reference for comparisons. Kaplan–Meier analysis was used to evaluate survival experiments, followed by a two-sided log-rank test, or a two-sided Wilcoxon test, which assigns greater weight to early time points, representing the bulk of the population.

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** All relevant data are available from the corresponding author. RNAseq raw data are available at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE97934.

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M.B. designed, performed and analyzed experiments, and contributed to writing; D.M.
designed, performed and analyzed experiments, and contributed to writing; J.C. per-
designed, performed and analyzed experiments; M.S. designed, performed and analyzed
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