**CHD8 regulates gut epithelial cell function and affects autism-related behaviors through the gut-brain axis**

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**Abstract**

Autism is a neurodevelopmental disorder characterized by early-onset social behavioral deficits and repetitive behaviors. Chromodomain helicase DNA binding protein (CHD8) is among the genes most strongly associated with autism. In addition to the core behavioral symptoms of autism, affected individuals also frequently present with gastrointestinal symptoms that are also common among individuals harboring mutations in the gene encoding CHD8. However, little is known regarding the mechanisms whereby CHD8 affects gut function. In addition, it remains unknown whether gastrointestinal manifestations may contribute to the behavioral phenotypes characteristic of autism. In the current study, we found that mice haploinsufficient for the large isoform of *Chd8* (*Chd8L*) exhibited increased intestinal permeability, transcriptomic dysregulation in gut epithelial cells, fewer tuft cells and goblet cells in the gut, and an overall increase in microbial load. Gut epithelial cell-specific *Chd8* haploinsufficiency was associated with an increase in anxiety-related behaviors, a phenotype that is often observed in autism and in cases of systemic *Chd8* haploinsufficiency, together with a decrease in tuft cell numbers. The antibiotic treatment of *Chd8L* haploinsufficient mice was also sufficient to attenuate social behavioral deficits. Together, these results thus suggest a pathway that may underlie autism-related GI deficits, while also providing a foundation for efforts to better understand how these deficits may play a direct role in the development of autism-related behaviors.

**Introduction**

Autism is a neurodevelopmental disorder characterized by early-onset social behavioral deficits and repetitive behaviors1. In addition to these core symptoms, many individuals with autism also experience a range of comorbidities. Gastrointestinal (GI) symptoms are common among patients with autism2, and can include constipation, bloating, abdominal pain, and diarrhea3. Constipation, for example, affects an estimated 20-33.9% of these patients4. Increases in GI problems have also been associated with the severity of autism5,6. In addition, many studies have reported that autism is associated with changes in the composition of the gut microbiome in both patients7–9 and in animal models of autism10.

In one study, an estimated 43% of individuals with autism reportedly exhibited abnormal intestinal permeability11, while a report published by De Magistris et al. revealed that autistic individuals (36.7%) and their relatives (21.2%) were more likely to exhibit abnormal intestinal permeability as compared with normal subjects (4.8%)12. Differences in intestinal permeability can be a direct result of changes in mucus secretion and/or the width of the mucus layer. Gut epithelial cells are primarily responsible for maintaining proper permeability and mucus production, the latter of which is mediated by the goblet cells in the gut epithelium13. However, little is known regarding the dysregulation or dysfunction of goblet cells in autism.

Chromodomain helicase DNA binding protein (*CHD8*) is one of the genes most strongly associated with autism14. Several studies have reported links between various severe *CHD8* mutations and the incidence of autism15–18. Encoded on chromosome 14q11.2, CHD8 is a chromatin remodeling factor19 that binds to β catenin20 and regulates the Wnt signaling pathway21. Wnt signaling is an intracellular pathway that is involved in many processes and has specifically been implicated in the regulation of proliferation during neurodevelopment22. Nearly 80% of patients harboring *CHD8* mutations have also been found to suffer from gastrointestinal problems, with 60% reporting specific issues such as constipation. Bernier et al. further found that interfering with *CHD8* expression in zebrafish results in slower gastrointestinal motility and a reduction in the number of post-mitotic enteric neurons15.

Despite these intriguing reports, little is known about the direct molecular mechanism through which CHD8 affects gut function. Notably, it remains uncertain as to whether changes in gut function may be related to autism-associated behavioral changes. To address this knowledge gap, we herein employed a mouse model harboring the heterozygous knockout of the large isoform of CHD8 (CHD8L) for studies of the gut-brain axis. While the total knockout of *Chd8L* has been reported to be lethal, Katayama et al. found that *Chd8L*+/- mice exhibit increased brain weight and brain volume relative to control mice, consistent with macrocephaly observed in autistic individuals with *CHD8* mutations15. *Chd8L*+/- mice also present with anxiety-like behaviors and deficits in social interaction, as characterized by a reduction in the duration of active contact relative to controls. These mutant mice did not have any differences in memory but did exhibit shorter intestines and slower GI motility23.

At present, the molecular basis for these GI changes has yet to be studied, and the connection between these alterations and autism-related behaviors remains unknown. In the current study, we thus sought to characterize differences in the gut function and molecular biology of *Chd8L*+/- mice, ultimately providing evidence that changes in GI characteristics may play a direct role in the anxiety-like effects that arise as a consequence of *Chd8* haploinsufficiency.

**Results**

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To initially explore the relationship between CHD8 and GI function, intestinal permeability was analyzed in *Chd8L*+/- and wild-type (WT) male mice following the validation of this experimental model. The behavioral phenotypes of these *Chd8L*+/- mice have previously been established, and include the dysregulation of social interactions together with anxiety-like behaviors24. The *Chd8L* haploinsufficiency evident in these mice was previously shown by Katayama et. al. to be sufficient to recapitulate the full behavioral phenotype seen in mice exhibiting total *Chd8* haploinsufficiency. In these *Chd8L*+/- mice, exons 11-13 of the *Chd8* gene are deleted. Immunohistochemical staining confirmed that CHD8 protein levels were reduced in the GI tract of these *Chd8L*+/- mice relative to WT controls (Fig. 1A). Consistently, real-time PCR analyses confirmed the significant downregulation of *Chd8* exons 11-13 in the gut epithelial cells of these haploinsufficient mice as compared to WT controls (Fig. 1B), whereas the expression of *Chd8* exon 1 increased in these *Chd8* haploinsufficient animals (Supplementary Fig. 1A). These results thus confirmed the downregulation of CHD8Lin these mice, with the upregulation of exon1 suggesting an attempt by these cells to compensate for the loss of CHD8L. Increased intestinal permeability has been reported in 36.7% of patients with autism as compared to just 4.8% of normal subjects12. To analyze intestinal permeability, mice were gavaged with FITC-dextran, and plasma levels of FITC Dextran were investigated at 2 or 6 h post-gavage. Plasma FITC-dextran levels were elevated in *Chd8L*+/- mice at 6 h post-gavage relative to WT controls (Fig. 1C), suggesting an increase in intestinal permeability in these animals. In contrast, there was no difference in stool transit time between the genotypes (Supplementary Fig 1B). In addition, a decrease in the length of the small intestine and the overall intestines was evident in these *Chd8L*+/- mice as compared to their WT counterparts, although no difference in colon length were observed between these groups (Fig. 1D). These results are consistent with the previous report by Katayama et al. revealing a decrease in intestine length in these genetically-modified mice23.

***Chd8L*+/- mice exhibit altered gut morphology**

The colonic mucus layer is the primary barrier that prevents lumenal antigens from encountering host tissues, and changes in this mucus layer can alter intestinal permeability. To analyze the gut morphology in these mice, we excised samples of small intestine and colon tissue for AB-PAS staining (Fig. 1E), which is mainly used to detect polysaccharides such as glycogen, glycoproteins, glycolipids, and mucins25. This approach revealed a reduction in mucus layer width in the colon of *Chd8L*+/- mice colon (Fig. 1F). A reduction in the number of mucus-producing goblet cells was also observed in the small intestine (Fig. 1G), although similar differences were not detected in the colon (Fig. 1H). No difference in villi length were detected in these animals (Fig. 1I). To understand whether these differences can also be detected at earlier developmental time points, we analyzed intestines from 4-week-old mice, revealing no differences in mucus layer width or goblet cell numbers between WT and *Chd8L+/-­* mice at this earlier time point (Supplementary Fig. 2). Staining for the tight junction marker ZO-1 in 8-week-old mice revealed no differences in tight junction morphology or ZO-1 intensity between these two groups of mice (Fig. 1J, K). These findings suggest that the observed reductions in goblet cell numbers and mucus layer thickness in *Chd8L+/-* mice may contribute to consequent increases in intestinal permeability.

**Transcriptomic analysis of gut epithelial cells from *Chd8L*+/- mice**

As CHD8 is a chromatin-binding protein, it likely impacts transcriptional activity in the gut. We thus performed whole transcriptomic sequencing using gut epithelial cells extracted from WT and *Chd8L*+/- mice. Strikingly, over 900 genes were differentially expressed between these two groups of mice, including 581 that were downregulated and 339 that were upregulated in *Chd8L*+/- gut epithelial cells (Fig. 2A, B; Supplementary Tables 1 and 2). Gene Ontology (GO) enrichment analyses indicated that the downregulated genes were enriched for markers of tuft cells, a subtype of gut epithelial cells, while upregulated genes were enriched for genes associated with immune cells and the immune response (Fig. 2 C, D, Supplementary Table 3). In particular, downregulated and upregulated genes were enriched for GO biological process terms related to mitochondrial function and the cell cycle, respectively (Fig. 2 E, F, Supplementary Table 3). Given the strong enrichment of tuft cell-related genes among downregulated genes for tuft cell markers, we verified this finding by comparing our downregulated gene list to a recently published list of subtypes of tuft cells identified by single-cell sequencing. Interestingly, 22 of our downregulated genes were among the list of 25 markers for type 2 tuft cells26. RT-PCR analyses further confirmed the downregulation of many tuft cell marker genes in samples from *Chd8L+/-* mice (Fig. 2G). Tuft cells are known to induce type 2 immune responses and to promote goblet cell development27. Reductions in tuft cells are thus consistent with the decreased goblet cell counts and mucus layer thinning observed above. RNA-seq analyses also indicated that two of the most highly upregulated genes in *Chd8L*+/- mice were the key antimicrobial peptides Reg3β and Reg3γ, as confirmed by RT-PCR (Fig. 2H). The decreased expression of tuft cell marker genes may reflect an overall drop in tuft cell numbers in these haploinsufficient mice. To test this possibility, immunohistochemical staining for the tuft cell marker protein DCLK1 was performed (Fig. 2I), revealing a decrease in DCLK1-positive cells in the small intestine of *Chd8L*+/- mice without any corresponding change in the colon (Fig. 2J).

***Chd8L*+/- mice exhibit an altered gastrointestinal microflora**

Given the potential role of the microbiota in autism and the increased antimicrobial peptide expression observed in *Chd8L+/-* mice, 16S rDNA sequencing was next used to compare the composition of the fecal microbiome between these animals and WT controls at 8 weeks of age. The total bacterial load in the small intestine and colon of these mice was initially assessed via PCR using 16S primers as previously reported28, revealing a significant increase in overall bacterial load in the colon of *Chd8L*+/- mice (Fig. 3A). Subsequent 16S rDNA sequencing revealed an increase in alpha diversity in the colon of *Chd8L*+/- mice (Fig. 3B), although no corresponding differences in beta diversity were evident between these genotypes (Fig. 3C). LEfSe analyses of these 16S sequencing data revealed three bacterial taxa that were differentially abundant between genotypes (Fig. 3D). Notably, *Chd8L+/-* mice exhibited an increase in the abundance of *Akkermansia muciniphila*, which has been widely implicated in the regulation of immune and neurological function. These *Chd8L*+/- mice thus display an increase in bacterial load, alpha diversity, and differences in select bacterial taxa within the gastrointestinal tract.

**Generation of gut epithelial cell-specific *Chd8* haploinsufficient mice**

Given the complex gut phenotype of *Chd8L*+/- mice, we decided to explore whether gut dysfunction can play a role in autism-related behavior. To answer this question, *Chd8* was specifically knocked in gut epithelial cells using a Cre-lox system, with the expression of Cre under the control of the gut epithelium-specific villin promoter. In all experiments, results were compared between *Chd8* gut epithelial haploinsufficient mice (Villin-Cre/Chd8flx+/-) and WT littermate controls. Knockout was validated by immunostaining of the gut of CHD8+/ΔIEC and WT mice (Fig. 4A). Knockout was further quantitively validated by RT-PCR. *Chd8* mRNA levels were significantly reduced in significantly downregulated in the CHD8+/ΔIEC mice (Fig. 4B).

In an open field test, *Chd8*+/ΔIEC mice exhibited fewer visits to the center (Fig. 4C) and decreased distance moved in the center (Fig. 4D), suggesting anxiety-like phenotypes, while total distance moved was comparable between these animals and WT controls (Fig. 4E). In an elevated plus maze test, these *Chd8*+/ΔIEC mice also spent less time in the open arms of the maze (Fig. 4F) and moved less distance in these open arms (Fig. 4G), further indicating anxiety-like behavioral phenotypes. These changes were also supported by a reduction in marble-burying by *Chd8*+/ΔIEC mice relative to WT controls (Fig. 4I). The absence of any change in grooming time suggests that these mice do not exhibit repetitive behaviors (Fig. 4J). In a three-chamber social behavior test, these mice also performed comparably to WT controls (Fig. 4K), indicating that social behavioral phenotypes were unchanged in these mice. Cre expression alone had no impact on any of these tested behavioral phenotypes (Supplementary Fig. 4). In summary, anxiety-like behaviors were specifically induced by the knockout of *Chd8* in the gut epithelial cells.

***Chd8*+/ΔIEC mice exhibit reduced tuft cell numbers**

Given the decreases in tuft cell numbers evident in mice exhibiting systemic *Chd8* haploinsufficiency, immunohistochemical staining was performed to detect tuft cells in the gastrointestinal epithelium of *Chd8*+/ΔIEC mice. A significant reduction in DCLK1-positive tuft cells was evident in both the small intestine and colon of 8-week-old *Chd8*+/ΔIEC mice (Fig. 5A, B), whereas Cre expression alone did not affect tuft cell numbers (Supplementary Fig. 5). Gut-specific *Chd8* haploinsufficiency is thus sufficient to induce a drop in tuft cell numbers in the gut epithelium, although no corresponding differences between *Chd8*+/ΔIEC and WT mice were observed with respect to the length of the colon, small intestine, or whole gut (Supplementary Fig. 3B). The bacterial load was also comparable between WT and *Chd8*+/ΔIEC  mice (Supplementary Fig. 3A).

**Transcriptome analyses of the brains of *Chd8*+/ΔIEC  mice**

To gain more insight into the possible molecular mechanisms underlying the increased anxiety phenotypes evident in *Chd8*+/ΔIEC mice, transcriptomic analyses were performed using frontal cortex and amygdala samples collected from these animals and WT controls. In total, 35 genes in the frontal cortex and 2 genes in the amygdala were differentially expressed (FDR < 0.05) in *Chd8*+/ΔIEC  mice relative to the corresponding tissues from WT animals (Fig. 5C). Interestingly, levels of complement subunit 3 (C3), an antimicrobial factor that has been implicated in anxiety, were upregulated in the amygdala of these mice. A gene set enrichment analysis (GSEA) of the genes differentially expressed in the frontal cortex revealed the upregulation of the oxidative phosphorylation, interferon-gamma response, and complement pathways in this compartment (Fig. 5D). As such, the complement system was upregulated in both of these brain regions at the transcriptional level in *Chd8*+/ΔIEC  mice.

**Antibiotic treatment attenuates the impairment of social behavior in *Chd8L*+/- mice**

Given the high bacterial load detected in *Chd8L*+/- mice, we next explored whether treating these animals with antibiotics was sufficient to attenuate the observed autism-like phenotypes. Briefly, 5-week-old WT and *Chd8L*+/- mice were administered a combination of ciprofloxacin (0.04 g/L), metronidazole (0.2 g/L), and vancomycin (0.1 g/L) in their drinking water for 3 weeks before subsequent experimental analysis. The total bacterial load present in the stool of both WT and *Chd8L+/-* mice was significantly reduced relative to the corresponding untreated animals, as measured by RT-PCR (Fig. 6A). In a three-chamber social interaction test, WT mice exhibited a preference for stranger mice over the empty cage irrespective of their antibiotic treatment status (Fig. 6B), as measured based on the time spent in each chamber. While untreated *Chd8L*+/- mice did not exhibit any preference for stranger mice, consistent with social behavioral defects, this deficit was attenuated following antibiotic treatment (Fig. 6C). WT animals also spent more time sniffing stranger mice relative to the empty chamber with or without antibiotic treatment (Fig. 6D). In contrast, untreated *Chd8L*+/- mice did not exhibit any difference in sniffing time between the empty chamber and unfamiliar mice, while following antibiotic treatment they spent significantly more time the unfamiliar mice relative to the empty chamber (Fig. 6E). In a dark-light test, *Chd8L*+/- mice also spent less time in the open arms relative to WT controls (Fig. 6F), in line with prior results23. However, no difference was observed between *Chd8L*+/- and WT control mice after antibiotic treatment (Fig. 6F). No differences between groups were observed in open field tests (Fig. 6G) or elevated plus maze tests (Fig. 6H). As such, these results suggest that antibiotic treatment was able to reverse some of the phenotypic changes evident in *Chd8L*+/- mice, with a particularly strong impact on social phenotypes and one indication associated with an anxiety phenotype.

**Discussion**

An association between GI symptoms and autism has long been documented, with a reported increase in the odds of developing these symptoms with greater autism severity5. Individuals harboring *CHD8* mutations also frequently exhibit a range of GI symptoms15. In the present study, we sought to explore the relationship between *CHD8* haploinsufficiency and GI dysfunction in mice, while also exploring the potential link between these factors and autism-related behavioral phenotypes. These *Chd8L* haploinsufficient mice exhibited an increase in intestinal permeability consistent with that observed in patients with autism12. The mucus layer is, in part, responsible for maintaining the integrity of the intestinal barrier29,30. We found that the width of the mucus layer was decreased in *Chd8L*+/- mice, with a concomitant reduction in the number of goblet cells in the small intestine of these mice at 8 weeks of age. Given that goblet cells produce mucus29, a decrease in goblet cell numbers can reduce the size of the mucus layer, in turn contributing to higher intestinal permeability. However, this reduction in goblet cell numbers was only evident in the small intestine whereas the thinning of the mucus layer was observed in the colon. While it is not clear as to whether a decline in the goblet cell population in the small intestine can directly influence mucus in the colon, it is possible that mucus levels in the small intestine are also reduced in these animals, although this is not technically possible to measure. These changes in GI morphology were consistent with the lower villi length evident in *Shank3*-knockout mice10.

In our study, we found that *Chd8L*+/- mice exhibited a higher bacterial load and greater alpha diversity in the colon. The functional consequences of this increase in alpha diversity are not clear. However, increases in microbiome richness may be related to the overall increase in bacterial load seen in these mice. The observation that these mice exhibited an overall increase in bacterial load is also somewhat novel, in part because most research focused on the microbiome primarily assesses microflora diversity via 16S sequencing without any corresponding quantification of bacterial load. Changes in bacterial load may thus have been overlooked in many previous studies. Transcriptomic analyses and RT-PCR revealed high levels of expression for antimicrobial peptides including Reg3β and Reg3γ in the gut epithelial cells of *Chd8L*+/- mice. The upregulation of these antimicrobial peptides may in part serve to compensate for the higher bacterial load in these mice.

While few changes in bacterial taxa were abundant when comparing murine genotypes, an increase in *A. muciniphila* was observed. Notably, *A. muciniphila* has previously been proposed as a possible probiotic owing to its positive effects on intestinal permeability31, although it can also reportedly cause colitis in some settings. Multiple studies have also detected elevated *A. muciniphila* levels in the context of neurological conditions including Parkinson’s Disease, Multiple Sclerosis, and autism spectrum disorders32. Further research is thus warranted to understand the precise link between this species and neurological pathogenesis.

Through transcriptomic analyses of the gut epithelial cells in *Chd8L*+/- and WT mice, several immune system-related genes were found to be upregulated in the haploinsufficient animals, whereas tuft cell marker genes were downregulated. Intriguingly, a prior RNA-seq analysis of the brains of adult mice of the same strain detected just five differentially expressed genes23, whereas we found over 900 differentially expressed genes in the gut. This suggests that CHD8 may have particularly important functions specifically in the gut during adulthood. Tuft cells are known to induce type 2 immune responses and are a source of IL-25, which drives a feed-forward signaling pathway involving tuft cells and type 2 innate lymphoid cells (ILC2s). These ILC2s serve as a source of IL-5, IL-9, and IL-13, which contribute to type 2 inflammation. IL-13 has also been shown to drive an increase in goblet cell numbers33. Reductions in tuft cell numbers may thus contribute to a concomitant decrease in goblet cell abundance, thereby reducing the width of the mucus layer in these mice.

One of the major behavioral phenotypes of *Chd8L*+/- mice first reported by Katayama et al. is an increase in anxiety-related behaviors24. Strikingly, the specific deletion of *Chd8* from gut epithelial cells in CHD8+/ΔIEC mice was sufficient to recapitulate the anxiety-like behaviors of *Chd8L*+/- mice exhibiting systemic *Chd8L* haploinsufficiency. This suggests that CHD8 in the gut epithelial cells plays a specific role in anxiety-like behavior, whereas it may not be relevant for social behaviors. Since approximately 30% of individuals with *CHD8* mutations display increased anxiety, our data suggests that this phenotype may be particularly related to the dysregulation of the gut15. It is possible that other nearby cell populations, such as gut immune cells or the enteric nervous system, play a role in regulating social behavior. In addition, these CHD8+/ΔIEC mice did not exhibit any differences in bacterial load. This further suggests that differences in bacterial load are more closely related to social behavior, as supported by the effects of antibiotic treatment on social behavior in *Chd8L*+/- mice.

Even though we did not detect any differences in GI morphology in *Chd8*+/ΔIEC mice, we did find that they exhibited fewer tuft cells in the colon and small intestine relative to WT controls. This is in contrast to findings from *Chd8L*+/- mice, in which tuft cell numbers were only reduced in the small intestine but not the colon. Given that tuft cell numbers are highly regulated by local microenvironmental conditions, including immune cells, it is possible that lack of CHD8 in the surrounding tissues masked the effects of epithelial cell *Chd8* haploinsufficiency on colonic tuft cell numbers.

To gain insight into the molecular mechanisms underlying the anxiety-related phenotype in *Chd8*+/ΔIEC mice, we performed an RNA-seq analysis of samples from both the frontal cortex and amygdala. In the amygdala, these animals exhibited significantly increased *C3* levels and decreased *Dock4* expression. This is interesting in light of the role of C3 in innate immunity, evidence from studies indicating that C3-knockout mice exhibit reduced anxiety34, and the finding that Alzheimer’s disease model mice experienced a drop in anxiety when treated with complement inhibitors35. While the overall number of changes in the amygdala was relatively limited, this observed increase in C3 expression may represent a particularly relevant finding. The upregulation of the complement pathway and other immune pathways in the frontal cortex transcriptome in *Chd8*+/ΔIEC mice further suggests that changes in the immune system function may be involved in anxiety-related behavior. The *Dock4* gene has been linked to the risk of autism, and *Dock4*-knockout mice reportedly exhibit autism-related behaviors36. Interestingly, *Dock4* has been characterized as a regulator of goblet cell differentiation and MUC2 production37. While these results are promising, the precise mechanisms through which tuft cell changes and gastrointestinal dysregulation may contribute to changes in the brain transcriptome and associated anxiety-related phenotypes in *Chd8L*+/- mice remain somewhat unclear. The dysregulation of immune-related factors may explain this link between changes localized to the gut and consequent behavioral alterations. This is supported by the known role that tuft cells play in type 2 immunity together with the increased expression of immune-related genes in the gastrointestinal tract of *Chd8L*+/- mice. Alternatively, an increase in gastrointestinal permeability may allow anxiogenic metabolites to enter the bloodstream and thereby reach the brain. The anxiety phenotypes in these animals may also be a result of pain or discomfort caused by changes in autonomic function38,39. Another limitation of this study is the fact that these analyses were restricted to male mice, and it is thus possible that there are sex-specific differences that we did not detect herein.

The gut microbiota has been associated with autism-related behaviors.4. For example in a three-chamber sociability test, germ-free (GF) mice spend more time in an empty chamber than in a chamber containing control mice relative to control animals4. Consistently, another study has demonstrated that GF mice spend more time with novel mice than controls40. In a murine model of autism, the administration of a single bacterial strain such as *Bacteroides fragilis* can reportedly increase social communication in the form of vocalizations, reduce anxiety, and attenuate repetitive behaviors, although it has no impact on behavior in a three-chamber social interaction test41. Microbial transfer has also been found to alleviate certain autism-related symptoms in individuals with autism42.

The findings of this study lend some support to previous evidence suggesting that a subset of individuals with autism may benefit from antibiotic treatment. Sandler et al. (2000), for instance, found that patients affected by the regressive onset of autism, as defined by normal development up to two years of age, followed by the onset of autism-associated behaviors, exhibited short-term improvements upon oral vancomycin treatment43. In another study, children diagnosed with autism displayed improved SCERTS Assessment Process Observation (SAP-O) scores at six months after treatment with antibiotics including amoxicillin and zithromycin44. In line with these results, the treatment of *Chd8L*+/- mice was associated with the abrogation of the reduced social behavior exhibited by these animals. Additional research should thus be conducted to determine whether individuals with autism caused by specific genetic etiologies may respond well to antibiotic treatment. Given that the underlying causes of autism may vary substantially among individuals, further research should focus on whether individuals with a specific genetic background or behavioral phenotypes are particularly responsive to antibiotic treatment.

In conclusion, *Chd8L* haploinsufficiency causes changes in GI morphology, tuft cell numbers, intestinal permeability, bacterial load, and alpha diversity in mice. Specifically knocking out *Chd8* in gut epithelial cells (CHD8+/ΔIEC) can cause anxiety-like behaviors without any corresponding changes in social behavior. Treating *Chd8L*+/- mice with antibiotics can rescue abnormal social behaviors in these mice. These results suggest that GI abnormalities may thus play a role in the symptomology and behavioral phenotypes of autism.

**Figure legends**

**Figure 1:**

**A**. Representative images of CHD8 staining in Wild type (WT) and *Chd8L*+/- mice. Staining for Chd8 (green) was performed, with Hoechst (blue) as a nuclear counterstain. The magnified area is marked with a red box. **B**. Real-time PCR analyses of *Chd8* exons 11-13, revealing a reduction in relative Chd8 levels in *Chd8*+/- mice (\*p < 0.05, two-tailed unpaired t-test; WT: n=5, *Chd8L*+/-: n=7). **C.** Intestinal permeability was measured based on plasma FITC-dextran levels at 2 and 6 h post-gavage (\*p < 0.05; WT: n=10, *Chd8L*+/-: n=15). **D**. Colon, small intestine, and whole gut lengths were compared between WT (n=6) and *Chd8L+/-* (n=9) mice (\*\*p < 0.01). **E.** Representative periodic acid Schiff staining results for WT and *Chd8L*+/- mice, with goblet cells indicated using red arrows and red lines corresponding to the width of the mucus layer. **F-I**. Morphological analysis mucus layer width (F), goblet cell number per villus (G), goblet cell number per crypt (H), and villi length (I), revealing a significant decrease in the number of goblet cells/villi (\*\*p < 0.01, Two-tailed unpaired t-test; WT: n=8, *Chd8L*+/-: n=6) and a significant reduction in mucus layer width (\*p < 0.05, two-tailed unpaired t-test; WT: n=35, *Chd8L*+/-: n=30). **J-K**. Immunohistochemical staining of colon and small intestine samples from WT (n=5 each) and *Chd8L*+/- (n=5, colon; n=4, small intestine). Representative ZO-1 staining results are shown (J) together with mean ZO-1 intensity (K). Data are means ± SEM

**Figure 2.**

**A**. A volcano plot highlighting differentially expressed gene distributions in *Chd8L*+/- gut epithelial cells. Each point represents genes in *Chd8L*+/- mice plotted against the level of statistical significance (−log10 adjusted p-value) and fold-change (log2 (*Chd8L*+/- vs. WT). **B.** Heatmap showing the 920 differentially expressed genes in *Chd8L*+/- mice gut epithelial cells compared to WT littermates (FDR-adjusted p ≤ 0.05). **C-F.**  [Gene Ontology](https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/gene-ontology) analyses of differentially expressed genes, including co-expression analyses of downregulated (C) and upregulated (D) genes, as well as biological process term enrichment results for upregulated (E) and downregulated (F) genes. **G.** Real-time PCR analyses of tuft cell markers including *Dclk1* (\*\*p<0.01), *Matk1* (\*\*\*p<0.001), *Clec4a* (\*\*p<0.01), and *Gnat3* (\*p<0.05) (Unpaired two-tailed t-test, WT: n=5, *Chd8L*+/-: n=7). **H.** Real-time PCR analyses of the antimicrobial peptide genes *Reg3*γ (\*p=0.05), *Reg3*β (\*p<0.05) (Unpaired two-tailed t-test, WT: n=5, *Chd8L*+/-: n=7). **I.** Representative immunohistochemical staining images of colon and small intestine tissue samples from analyses of WT (n=4) and *Chd8L*+/- (n=5) mice. Samples were stained for DCLK1 (green) with Hoechst (blue) as a nuclear counterstain. **J.** Significant decreases in the numbers of tuft cells per mm2 were evident in the small intestine of *Chd8L*+/- mice relative to WT mice (\*\*\*p < 0.001; unpaired two-tailed T-test). Data are means ± SEM.

**Figure 3.**

**A.** Relative Bacterial load in the small intestine and colon of WT and *Chd8L*+/- mice (\*p<0.05, unpaired two-tailed t-test; n=8/group). Data are means ± SEM. **B**. Measures of alpha diversity revealed significantly increased evenness in *Chd8L*+/- mice relative to WT mice (\*p<0.05, Kruskal-Wallis pairwise analysis, n=8/group). **C**. Weighted UniFrac-based PCoA plot-based visualization of the microbial communities in all *Chd8L*+/- and WT mice. **D.** Bacterial taxa that were overrepresented (red) or underrepresented (green) in *Chd8L*+/- mice, as identified according to LefSe analyses of 16S high-throughput sequencing data.

**Figure 4.**

**A**. Representative CHD8 staining results from wild-type (WT) and *Chd8*+/ΔIEC mice. Samples were stained for CHD8 (green), with Hoechst as a nuclear counterstain (blue). Behavioral testing results were compared between WT mice and those exhibiting *Chd8* haploinsufficiency specifically in epithelial cells (*Chd8*+/ΔIEC). **B**. Relative quantification of *Chd8* exon 3 was performed via real-time PCR, revealing a significant reduction in *Chd8*+/ΔIEC mice. (\*p<0.05, unpaired two-tailed t-test; n=6 per genotype). **C-E.** Open field test results. **C.** Visits to the center were significantly reduced for *Chd8*+/ΔIEC mice (n=13) relative to WT controls (n=14) (\*p<0.05, unpaired two-tailed t-test). **D.** Distance moved in the center was significantly reduced for *Chd8*+/ΔIEC mice (n=13) relative to WT controls (n=10) (\*\*p<0.01, unpaired two-tailed t-test). **E.** Distance moved in the arena, revealing no significant differences between groups (p=0.8). **F-H.** Elevated plus maze test results. **F.** *Chd8*+/ΔIEC mice spent less time in the open arms of the maze (\*p<0.05, unpaired two-tailed t-test; WT: n=14, *Chd8*ΔIEC: n=11). **G.** *Chd8*+/ΔIEC mice moved less distance in the open arms of the maze ( \*p<0.05, unpaired two-tailed t-test; WT: n=14, *Chd8*ΔIEC: n=12). **H.** No significant differences in the distance moved in the arena were observed among groups (p=0.1). **I.** In a marble burying test, *Chd8*+/ΔIEC mice buried significantly fewer marbles than WT mice (\*\*p<0.01, unpaired two-tailed t-test; WT: n=14, *Chd8*ΔIEC: n=12). **J.** No significant difference in grooming time was observed between groups (p>0.05). **K.** In a social preference test, both WT and *Chd8*+/ΔIEC mice showed a preference for stranger mice. (\*p<0.05, two-way ANOVA with Tukey’s post-hoc test; WT: n=15, *Chd8*+/ΔIEC: n=13). Data are means ± SEM.

**Figure 5.**

**A-B.** Immunohistochemical staining was performed using samples from 5 mice per group. Representative DCLK1 staining is shown for small intestine and colon samples (A), with corresponding quantification of the numbers of tuft cells per mm2 (B) in these tissue compartments (\*p<0.05, \*\*\*p<0.001; unpaired two-tailed t-test, n=5/group). Data are means ± SEM. **C**. Volcano plot highlighting differentially expressed gene distributions in *Chd8*+/ΔIEC brain regions including the frontal cortex and amygdala. Each point represents genes in *Chd8*+/ΔIEC mice plotted against the level of statistical significance (−log10 adjusted p-value) and fold-change (log2 (*Chd8*+/ΔIEC vs. WT). **D**. GSEA analyses indicating enriched gene pathways in the frontal cortex based on the fold-change difference in gene expression between the WT and *Chd8*+/ΔIEC groups.

**Figure 6.**

**A**. Real-time PCR analyses of relative bacterial load following antibiotic treatment, revealing a significant reduction in bacterial load in antibiotic-treated WT mice relative to WT mice and in antibiotic-treated *Chd8L*+/- mice relative to *Chd8L*+/- controls. (\*p<0.05, Two-way ANOVA with Tukey’s post-hoc test; n=8/group). **B-C.** Social preference tests were performed. **B.** WT control (n=12) and antibiotic-treated WT (n=11) mice exhibited a preference for stranger mice (Two-way ANOVA with Tukey’s post-hoc test). **C**. *Chd8L*+/- control mice (n=14) did not exhibit any preferences, whereas antibiotic-treated *Chd8L*+/- mice (n=13) exhibited a preference for stranger mice (Two-way ANOVA with Tukey’s post-hoc test). **D.** WT control (n=11) and antibiotic-treated WT (n=12) mice spent more sniffing stranger mice (Two-way ANOVA with Tukey’s post-hoc test). **E.** No significant differences in time spent sniffing an empty chamber or stranger mice for *Chd8L*+/- control mice (n=8), whereas antibiotic-treated *Chd8L*+/- mice (n=10) spent more time sniffing strangers mice than the empty chamber (Two-way ANOVA with Tukey’s post-hoc test). **F**. Dark light test results revealed that *Chd8L*+/-control mice (n=8) spent significantly less time in the light zone (\*p<0.05, Two-way ANOVA with Tukey’s post-hoc test; antibiotic-treated *Chd8L*+/- mice: n=9.) **G.** Time spent in the center in an open field test. **H.** Time spent in the open arms in an elevated plus maze test, revealing no significant differences among groups. Data are means ± SEM.

**Supplementary Figure 1.**

**A**. Real-time PCR analyses of *Chd8* exon 1. Relative *Chd8* quantities significantly increased in *Chd8L*+/- mice (\*p < 0.05, two-tailed unpaired t-test; WT: n=5, *Chd8L*+/-, n=7). **B**. Transit time assay (p > 0.05, two-tailed unpaired t-test; WT: n=6, *Chd8L*+/-, n=4). Data are means ± SEM.

**Supplementary Figure 2.**

**A.** Representative periodic acid Schiff staining results from 4-week-old WT and *Chd8L*+/- mice. **B.** Numbers of goblet cells per villi (p > 0.05). **C**. Villi length (p > 0.05). Data are means ± SEM.

**Supplementary Figure 3.**

**A.** Relative Bacterial load in the small intestine and colon of *Chd8*+/ΔIEC and WT mice. (p > 0.05, two-tailed unpaired t-test; WT: n=10, *Chd8*+/ΔIEC, n=7). **B.** Colon, small intestine, and whole gut lengths in WT and CHD8+/ΔIEC mice (n=5 per group). Data are means ± SEM.

**Supplementary Figure 4.**

**A-C.** Open field test. **A.** No differences in visits to the center were observed among groups (p > 0.05; unpaired two-tailed t-test; WT: n=13, Vil cre-positive: n=10). **B.** No differences in distance moved in the center were observed among groups (p > 0.05; unpaired two-tailed t-test; WT: n=13, Vil cre-positive: n=10). **C.** No differences in distance moved in the arena were observed among groups (p > 0.05). **D-F.** Elevated plus maze test. **D.** No differences in time spent in open arms were observed among groups (p > 0.05; unpaired two-tailed t-test; WT: n=12, Vil cre-positive: n=9). **E.** No differences in distance moved in open arms were observed among groups (p > 0.05; unpaired two-tailed t-test; WT: n=13, Vil cre-positive: n=8). **F.** No differences in distance moved in the arena were observed among groups (p > 0.05; unpaired two-tailed t-test; WT: n=13, Vil cre-positive: n=10). **G.** Marble burying test results (p > 0.05; unpaired two-tailed t-test; WT: n=13, Vil cre-positive: n=10). **H.** Social preference test results revealed that both WT and Vil cre-positivemice exhibited a preference for stranger mice (\*p<0.05; Two-way ANOVA with Tukey’s post-hoc test; WT: n=13, Vil cre-positive: n=10). Data are means ± SEM.

**Supplementary Figure 5.**

**A.** Representative DCLK1 staining images for small intestine and colon samples from WT and Vil cre-positive mice. **B.** Numbers of tuft cells per mm2 in the small intestines and colon. p > 0.05, n=5 for both genotypes. Data are means ± SEM.

**Materials and methods**

**Mice:** All mice were bred and maintained in animal facility of faculty of medicine, Bar Ilan University and experimental procedures were approved by Institute Animal Ethical Committee. All mice in a vivarium at 22°C in a 12-hr light/dark cycle, with food and water available ad libitum. In this manuscript, we used the C57BL/6 CHD8L +/- mice, described in *Katayama et al*23, which have been previously phenotyped. These mice were kindly provided by Keiichi I. Nakayma, through Riken. C57BL/6 Chd8L+/- male mice were crossed with female wild type mice to produce wild type and C57BL/6 Chd8L+/- mice. To generate gut epithelial cell specific CHD8 haploinsufficiency, male Villin-Cre+/- mice were crossed with female CHD8fl/fl mice. The floxed CHD8 mice was purchased from Jackson Laboratories (stock number 031555). Therefore, all offspring were CHD8fl/wt, while half the offspring were Villin-cre +/- (Villin cre positive). Cre-negative (wild type) and Cre-positive (CHD8 floxed heterozygote) littermate offsprings were used in all experiments, haploinsufficient mice were referred to asCHD8+/ΔIEC. The test mice used without the studies were always the first-generation offspring of the above-mentioned breeding schemes. All behavioral tests were performed with 8 to 10 weeks old mice. Only male mice were used throughout this study.

**Gut-Permeability assay:**

8 weeks old mice were fasted for 6 hr, and then administrated 14 ml/kg body weight of phosphate-buffered saline (pH 7.4) containing 22 mg/ml fluorescein isothiocyanate conjugated dextran (FITC-dextran, molecular mass 4.4 kDa; Sigma Chemical, St.) by gavage. A blood sample (150 µl) was obtained in a capillary tube 2 hr and 6hr after administration of the markers by orbital retro bulbar puncture. The blood samples were centrifuged (3,000 rpm at 4°C) for 15 min. Plasma (50 µl) was mixed with an equal volume of phosphate-buffered saline (PBS; pH 7.4) and added to a 96-well microplate (black). The concentration of FITC-Dextran was determined by spectrophotometry with an excitation wavelength of 485 nm (20 nm band width) and an emission wavelength of 530 nm (25 nm band width) using serially diluted samples of the marker as standard.

**Histology:**

Small intestine and colon were excised from 8 weeks old mice, immediately submerged in Ethanol-Carnoy’s Fixative at 4°C for 2 hr and then placed into 100% ethanol and subsequently in 50%, 75% and 100% xylene and then embedded in paraffin. It was then cut into 5 µm sections.

Alcian-blue Periodic acid sciff's reagent (Ab/PAS) staining: The tissue sectioned were deparaffinised and stained with alcian blue (Sigma; A5268) for 15 minutes. After washing with distilled water, it was treated with periodic acid (Sigma Aldrich; p7875) for 5 minutes, followed by washing in distilled water for 3 minutes, stained with schiff's reagent (Sigma Aldrich, 3952016) for 10 minutes. Then it was washed under running tap water for 5 minutes and nuclei were stained with haematoxylin for 1 minute. Sections were then dipped into acid alcohol and then dehydrated and mounted. Number of goblet cells and length of mucus layer was measured using ZEN Desk software.

**Immuno-staining:**

Sections were deparaffinised followed by antigen retrieval with Sodium Citrate buffer (pH=6) for 20 minutes at 60°C. After cooling down it was permeabilized in 0.1% Triton X-100 for 10 minutes. Blocking was done in 2% BSA (Calbiochem, 126575) and 1% goat serum in 0.1% Tris buffer saline with triton-X (TbTx) for 3hr at room temperature. The tissue sections were then subjected to immunofluorescence staining with following anitbodies, CHD8(1:100) antibody (abcam: ab84527), ZO-1 (1:200) antibody (Thermo Fisher Scientific; 40-2200), DCLK1 (1:200) antibody (abcam: ab31704) for 1hr at room temperature and followed by overnight incubation in 4°C. The cells were then washed with cold TbTx three times for 10 min each, and incubated with Alexa 488-labeled anti-rabbit secondary antibody (1:200) (Jackson immune research laboratories 111-545-144) at room temperature for 2.30 hr. Then after washing with TbTx 3 times 10 minutes each, they were stained with Hoechst (sigma) (1:1000) while mounting. The sections were examined by fluorescence microscopy.

**Stool collection, DNA extraction and sequencing of 16s rRNA gene**

Mice fecal samples were collected directly from the small intestine and colon by scrapping and stored at -80 oC until further analyses. DNA was isolated using the PureLinkTM Microbiome DNA Purification Kit according to the manufacturer's instructions. The V4 region of bacterial 16S rRNA gene was PCR-amplified using the 515F and 806R primers45. Forward primers included unique 12-base barcodes in order to tag PCR products from different samples. PCR reaction consisted of PrimeSTAR Max Premix 1x (Takara Bio), 0.4 μM of each primer and 30-100 ng DNA template. Reaction conditions were as following: initial denaturing step for 3 min at 95oC, followed by 30 cycles of 10 s at 95oC, 5 s at 55oC and 5 s at 72oC. PCR reactions were performed in duplicates for each sample, pooled and purified with Agencourt AMPure XP kit (Beckman Coulter). Purified PCR products were quantified using a Qubit dsDNA HS assay kit (Life Technologies) and 50 ng of each sample was pooled for further sequencing on the Illumina MiSeq platform.

**Bioinformatic analyses of 16S rRNA gene sequences**

Obtained 16s rRNA sequencing data were analyzed by QIIME 1 pipeline 46. Alpha diversity (within community diversity) was estimated by Gini coefficient, as a measure of community evenness. Beta diversity (between communities diversity) was calculated using weighted UniFrac distances. The diversity parameters were compared between groups using a nonparametric t-test with Monte Carlo permutations (999) to calculate p values, and Benjamini and Hochberg FDR method was used afterwards to correct p values for multiple comparisons between different pairs of groups. Linear discriminant analysis (LDA) effect size (LefSe) was used to identify differences in relative abundance at different taxonomic levels. Values used in analysis were alpha = 0.05 and LDA threshold of 2.0.

**Epithelial Cell extraction from gut:**

Epithelial cells were isolated as previously described by Zeineldin et al47. Intestinal pieces were washed with PBS, followed by treatment with 0.04% sodium hypochloride for 15 minutes on ice. Then intestinal pieces were put in solution B (2.7 mM KCL, 150mM NaCl, 1.2mM KH2PO4, 680 Mm Na2HPO4, 1.5mM EDTA, 0.5mM DTT) for 15 minutes. Then solution B was discarded and the pieces were put in PBS followed by vortex for 50 seconds. This was repeated 3 times and then the solution was centrifuged at 1000 g, 10 min at 4oC. Epithelial cells are collected in the pellet. Epithelial Cells were collected from 5 wild type and 7 CHD8L+/- mice. All mice were eight week old male littermates. Collected pellet were resuspended Buffer RLT for RNA purification using the RNeasy Micro kit (Qiagen 74004) following the standard protocol with on-column DNase digestion.**RNA extraction from different brain regions:**

Mice were sacrificed by rapid decapitation and brains were quickly removed. The frontal cortex and amygdala samples were isolated using brain matrix and gauge 13 and immediately frozen on dry ice. Total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol.

**RNA sequencing and analysis (gut epithelial cells and brain regions):**

Sequencing libraries were prepared using NEBNext Poly(A) mRNA Magnetic Isolation Module & NEBNext® Ultra™ II RNA Library Prep Kit for Illumina® sequencing. The 75 base pair single end sequencing was carried out on the Nextseq 75SR. Reads were mapped to the Mus Musculus reference genome (mm10) using the Tophat2 software (release Tophat2.0.12). Differential expression file for gut epithelial cells can be found in supplementary table 1 and mapped reads can be found in supplementary table 2. Differentially expressed genes and mapped read files for frontal cortex and amygdalar RNA-seq is found in supplementary table 6.Differential gene expression analysis was carried out by using DESeq2 pipeline. Enrichment analyses for the Gene ontology (GO) terms (biological process and co-expression atlas) were performed using online ToppGene Suite software. GO terms were considered to be significant when the Benjamini and Hochberg FDR adjusted p value was below 0.05. Raw data and read count data from this analysis are available at GSE182815. GSEA analysis was performed on lists of genes from the RNA-seq analysis that are ranked by their fold change between the two experimental groups. GSEA was performed as described previously48 with the use of GSEA v.2.0.1 (http://www.broadinstitute.org/gsea).

**Real time PCR:**

RNA was converted to cDNA using Maxima H minus first strand cDNA synthesis kit with dsDNAse (Thermo Scientific). Real time PCR for tuft cell markers, antimicrobial peptide genes, CHD8 exon 1, exon 11-13, exon 3 was performed using Fast Start Universal SYBR Green Master (Roche) and ViiA™7 Real-Time PCR System (Life Technologies). PCR consisted of 40 cycles, using melting temperature of 95 °C for ten seconds per cycle, and an annealing temperature of 60 °C of thirty seconds per cycle. Relative quantification by ddCt method was used to measure tuft cell abundance in the gut. The primer sequences used in the reactions are indicated in the Supplementary Table 4.

**Bacterial load quantification**:

Bacterial load quantification was performed as described previously by Nadkarni et al.28 using Taqman and a ViiA™7 Real-Time PCR System (Life Technologies). PCR thermocycler ssettings consisted of 40 cycles of 95°C for 20 s and 60°C for 20 s. The ΔΔCt method was used to quantify relative bacterial abundance. Primer and probe sequences used for these analyses are listed in Supplementary Table [4](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6456569/#MOESM1).

**Antibiotic Treatment:**

5 weeks old CHD8L+/- mice and their WT controls were given a combination of ciprofloxacin (0.04gl-1), metronidazole (0.2gl-1) and vancomycin (0.1 gl-1) in their drinking water for 3 weeks**.**

**Behavioral testing**: Mice were habituated to the room for at least 1 h before commencement of each test. Each test was performed on a separate day, usually with one day rest between each test. A camera films the movement, and the Noldus Software “ethovision” tracks the behavior of the animals.

**Open field test**: Mouse is placed in the corner of a plastic square box (50X50 cm) where it moves freely for ten minutes under ~120LUX of light. During this time, a camera films and tracks the behavior of the animals, including distance traveled.

**Light/dark box test**: The mouse is placed in a dark plastic chamber (75X75 cm) with an opening to highly lit chamber (~1200LUX). The mouse is free to move between the two chambers for five minutes. During this time, a camera films and tracks the behavior of the animals, including where they are found inside the box, velocity, distance travelled, etc

**Elevated plus maze**: The mouse is placed in the center of a four arms maze. Each arm is 30 cm in length, and two are closed and two are open. The maze is approximately one meter high. The mouse is free to choose which arm it enters for a five minute period. During this time, a camera films and tracks the behavior of the animals, including where they are found in the maze, velocity, distance travelled, etc.

**Social interaction test:** The test took place in a Non-Glare Perspex box (60X40 cm) with two partitions that divide the box to three chambers, left, center and right (20X40 cm). The mouse is placed in the middle chamber for habituation (5 min) when the entry for both side chambers is barred. Test mouse was then allowed to explore the whole arena (10 min), where they freely choose between interacting with a novel mouse in one chamber or stay in an empty chamber (social test). During this time, a camera films and tracks the behavior of the animals, including time spent in each chamber. For analysis of mouse sniffing, we analyzed the interaction between nose of test mouse with nose or body of stranger mouse. Interaction within an area of 2 mm was defined as positive sniffing interaction.

 **Marble burying test:** Repetitive marble burying was measured. The apparatus is a Non-Glare Perspex (20X40 cm). Twenty green glass marbles (15 mm in diameter) were arranged in a 4 X 5 grid that covered 2/3 of the apparatus on top of 5 cm clean bedding. Each mouse was placed in the corner that did not contain the marbles and was given 30 min exploration period, after which the number of marbles buried, was counted. “Buried” was defined as 2/3 covered by bedding. Testing was performed under dim light (25 lux).

**Self-Grooming**: Mice are scored for spontaneous self-grooming. Each mouse is placed individually into the open field chamber. After 10 minutes habituation period, each mouse was scored using parameters of cumulative time spent grooming during a 20 minutes session.

**Statistics:** Appropriate Statistical tests, two tailed unpaired T-test or two way anova has been performed as outlined in the manuscript and , were performed in graphpad prism 9.3 software. Data is presented as mean ± standard error of the mean. We have now added detail of all results of all statistics outlined in this manuscript, including which statistical test was used in each experiment, in Supplementary Table 5.

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