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Article

Role of TRP channels in shaping gut microbiome

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Abstract: Transient receptor potential (TRP) channel family proteins are sensors for pain, which sense variety of thermal and noxious chemicals. Sensory neurons innervating the gut abundantly express TRPA1 and TRPV1 channels and are in close proximity of gut microbes. Emerging evidence indicates a bi-directional gut-brain cross-talk in several entero-neuronal pathologies; however, the direct evidence of TRP channels interacting with gut microbial populations is lacking. Herein, we examine whether and how the knockout (KO) of TRPA1 and TRPV1 channels individually or combined TRPA1/V1 double-knockout (dKO) impacts the gut microbiome in mice. We detect distinct microbiome clusters among the three KO mouse models versus wild-type (WT) mice. All three TRP-KO models have reduced microbial diversity, harbor higher abundance of Bacteroidetes, and reduced proportion of *Firmicutes*. Specifically distinct arrays in the KO models are determined mainly by *S24-7*, *Bacteroidaceae*, *Clostridiales*, *Prevotellaceae*, *Helicobacteriaceae*, *Rikenellaceae*, and *Ruminococcaceae*. A1KO mice have lower *Prevotella*, *Desulfovibrio*, *Bacteroides*, *Helicobacter* and higher *Rikenellaceae* and *Tenericutes*; V1KO mice demonstrate higher *Ruminococcaceae*, *Lachnospiraceae*, *Ruminococcus*, *Desulfovibrio* and *Mucispirillum*; while A1V1dKO mice exhibit higher *Bacteroidetes*, *Bacteroides* and *S24-7* and lower *Firmicutes*, *Ruminococcaceae*, *Oscillospira*, *Lactobacillus* and *Sutterella* abundance. Also, the abundance of taxa involved in biosynthesis of lipids and primary and secondary bile acids is higher while that of fatty acid biosynthesis-associated taxa is lower in all KO groups. To our knowledge, this is the first study demonstrating distinct gut microbiome signatures in TRPA1, V1 and dKO models and should facilitate prospective studies exploring novel diagnostic/ therapeutic modalities regarding the pathophysiology of TRP channel proteins.

Keywords: Intestinal microflora; Microbiota; Pain; Transient Receptor Potential; TRP channels; TRPA1; TRPV1.

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1. Introduction

Transient receptor potential (TRP) channel family of proteins are known as a detector for various external and internal stimuli and react to an array of changes in temperatures, acidic and basic pH, osmolarity, odorants, chemicals, intracellular lipid mediators such as anandamide and lipoxygenase products, fatty acids and mechanical stimuli[1-3]. Among these, temperature sensation through TRP channels provides critical information about environment and triggers perceptual reflexes and responses ranging from sensation to pain. Hence, TRP receptors that sense thermal- and chemical-induced pain are an important receptor family involved in generation of pain. Expressed by primary afferent neurons, both TRPA1 (activated by extreme cold) and TRPV1 (major mammalian sensor of noxious heat) function as a sensor for detecting inside and outside painful stimuli, temperature and inflammation[4]. Neurons innervating the gut abundantly express TRPA1 and TRPV1 channels[5], and are in close proximity of highly diverse and complex community comprising trillion of microbes living in the intestinal tract (gut microbiome).

Recent and ever-mounting evidence has revealed the critical role of the gut microbiome in wide array of pathologies including, but not limited to, gastrointestinal, metabolic, cardiovascular, neurological, and psychiatric disorders[6]. However, data on the possible role of the gut microbiome in pain-related pathophysiology outside of the gastrointestinal tract is scarce. Given the rapidly emerging evidence related to the interactions between the gut microbiome and the central nervous system (CNS), also known as the 'gut–brain axis'[7], it is reasonable to hypothesize that the gut microbiome may also be linked to the physiology of pain modulation through the prominent pain receptors including TRPs[8-10]. Several animal studies have shown that the gut microbiome play an important role in the development of visceral pain[11,12] as well as the neuropathic pain[13]. Several human studies have also reported gut microbiome alterations in patients with several visceral pain disorders including irritable bowel syndrome (IBS)[14-16], chronic dysfunctional pelvic pain[17,18], chronic fatigue syndrome [19,20], rheumatoid arthritis and spondyloarthropathies[21,22], and fibromyalgia[23,24]. Despite these reports, there is still no evidence of such gut microbiome alterations in the milieus involving non-visceral pain. Further, the specific and precise interaction between gut microbiome and TRP channels remains unclear. Emerging evidence indicate that the communication between gut and brain is bidirectional[7]. Neuronal lines of communication help the brain to regulate and control the gastrointestinal processes, while signals relayed back by the GIT can influence both perception and host behavior[7]. Gut microbiome and its metabolites can influence gut-brain axis[25] to change sensation in peripheral nerves, and perception circuits in the brain. Similarly, brain and sensory neurons innervating the gut can also influence microbial communities; however, whether and how TRP channels can influence the gut microbiome remains unknown.

Most recently, the involvement of bioactive lipids, such as the N-acylethanolamine (NAE) family whose main members are N-arachidonoylethanolamine (AEA), palmitoylethanolamide (PEA) and oleoilethanolamide (OEA), as well as the short-chain fatty acids (SCFAs) such as butyrate in gut has been found to modulate peripheral and central neuronal processes[26]. More than 50 arachidonic acid- and linoleic acid-metabolites as well as lysophospholipids, and isoprenoids are among the endogenous TRP-channel sensitizers, activators and inhibitors, and modulates neuronal sensations[27-31]. Therefore, the lipid metabolism modulation in the gastrointestinal tract and the gut microbiome can influence the neuronal sensation and vice-versa. However, direct evidence of gut microbiome regulation by TRP channels is still lacking. Herein, we for the first time examine whether and how the individual knockout of TRPA1 and TRPV1 channels as well as the combined TRPA1/V1 double-knockout in mice impacts the gut microbiome and its metabolic pathways.

2. Results

2.1. Deletion of TRP-A1 and -V1 channels individually (KO) or both -A1/V1 together (dKO) distinctly impacts gut microbiome diversity

The analysis of β-diversity (a measure of microbial diversity differences among the groups) of the gut microbiome reveal that the three different mouse models of TRPA1, TRPV1 and TRPA1/V1

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dKO harbor distinct signatures of the gut microbiome as compared to their age- and gender-matched wild-type (TRP-WT) counterparts (Fig. 1a). The β-diversity of microbiome signatures of three KOs are clustered distinctly from each other as well as from WT counterparts. The microbiomes of V1 KO and A1/V1 dKO are clustered relatively close to each other while the A1 KO is relatively closer to WT but still clearly distinct from other groups (Fig. 1a; Suppl. Fig. 1a). Further analyses of α-diversity (a measure of microbial diversity within samples) indices (i.e., Chao1 [species richness], number of OTUs [operational taxonomic units] detected, phylogenetic diversity [PD] and Shannon index [species evenness]) also show that the three KOs mice harbor distinct populations of gut microbes indicated by significant differences in the α-diversity indices of the gut microbiome. The A1/V1 dKO mice show the lowest and most distinct pattern in terms of all α-diversity indices (Fig. 1b-e). Overall, the α-diversity in A1 KO mice remains similar to that in WT mice while the V1 KO mice show marginally reduced indices of PD whole tree, observed number of OTUs, and species richness (Chao1) and evenness (Shannon) as compared to WT and A1 KO counterparts (Fig. 1b-e). In contrast, A1/V1 dKO mice demonstrate remarkably reduced phylogenetic diversity, observed number of OTUs and species richness and evenness as compared to all of the other three groups of mice (Fig. 1b-e).

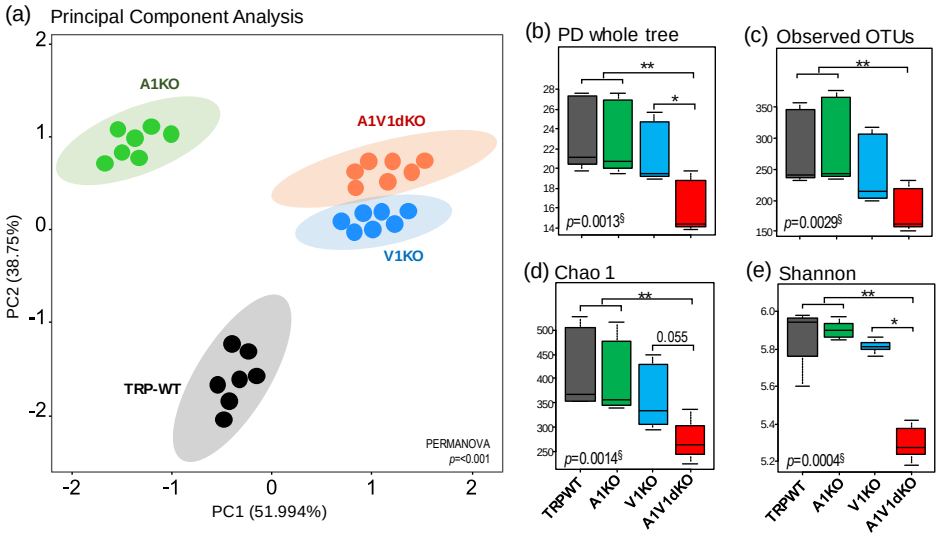

Figure 1. Distinct patterns of gut microbiome diversity in mouse models of TRPA1 knockout, TRPV1 knockout and TRPA1V1 double-knockout as compared to each other as well as to the TRP wild-type counterparts. (a) Beta-diversity (PCoA) plot of the gut microbiome in mouse models of TRPA1 knockout (A1KO), TRPV1 knockout (V1KO) and TRPA1V1 double-knockout (A1V1dKO) versus TRP wild-type (TRP-WT) counterparts. (b-e) Alpha-diversity indices i.e., PD whole tree (phylogenetic diversity), Observed number of operational taxonomic units (OTUs), Chao1 (species richness) and Shannon (species evenness) in mouse models of TRPA1 knockout (A1KO), TRPV1 knockout (V1KO) and TRPA1V1 double-knockout (A1V1dKO) versus TRP wild-type (TRP-WT) counterparts. §Kruskal-wallis test; *p<0.05, **p<0.001 (pair-wise Dunn's posthoc test).

2.2. Deletion of TRP-A1 and -V1 channels individually or both -A1/V1 together generates distinct microbiome composition in the mouse gut

The relative abundance of major phyla is found to be significantly distinct in three KO groups as compared to WT counterparts as well as to each other (Fig. 2a), suggesting that each of these three TRP-genotypes developed a unique microbial phyla signature. All three KO groups have increased proportion of phylum *Bacteroidetes* with proportion being significantly highest in A1/V1 dKO mice followed by V1KO while the proportion in A1KO mice is only marginally higher (Fig. 2a, c). Oppositely, the abundance of phylum *Firmicutes* demonstrates an inverse (of phylum *Bacteroidetes*) pattern characterized by lowest proportion in the dKO mice versus all of the other groups (Fig. 2a, b). Overall, the A1 KO mice show the highest ratio of *Firmicutes* to *Bacteroidetes* while the ratio in V1 KO mice is equivalent to that in WT mice, in contrast to the A1/V1 dKO mice that demonstrate the

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significantly lower ratio compared to all other three groups of mice (Fig. 2e). Furthermore, the abundance of the third major phylum i.e., *Proteobacteria* is remarkably diminished in all of the three KO groups compared to WT counterparts (Fig. 2d). The overall ratio of gram-positive and –negative bacteria is significantly higher in A1- and V1-KO but significantly lower in A1V1dKO mice compared to WT counterparts (Fig. 2f). In addition, all of the three KO models harbor remarkably higher ratio of obligate anaerobic bacteria over aerobic bacteria (Fig. 2g). Subsequent analysis of relative abundance at the level of bacterial families and genera also reveal specifically distinct and unique arrays in all the four groups wherein the differences in the KOs vs. WT groups are determined largely by the members of the families *S24-7*, *Bacteroidaceae*, unclassified *Clostridiales* family, *Prevotellaceae*, *Helicobacteriaceae*, *Rikenellaceae*, and *Ruminococcaceae* (Fig. 2h-i). The organism-level phenotype analysis reveal significantly higher proportion of OTUs corresponding to potential pathogenic bacteria in all the three KO models, with proportion being highest in A1V1dKO followed by V1KO and A1KO (Fig. 2j). Further, the proportion of potential biofilm forming bacteria is significantly lower in all the three KO models versus WT mice (Fig. 2k). In addition, the proportion of bacteria containing mobile elements is significantly lower while that of stress tolerant bacteria is higher in all the three KO groups versus WT counterparts (Suppl. Fig. 2).

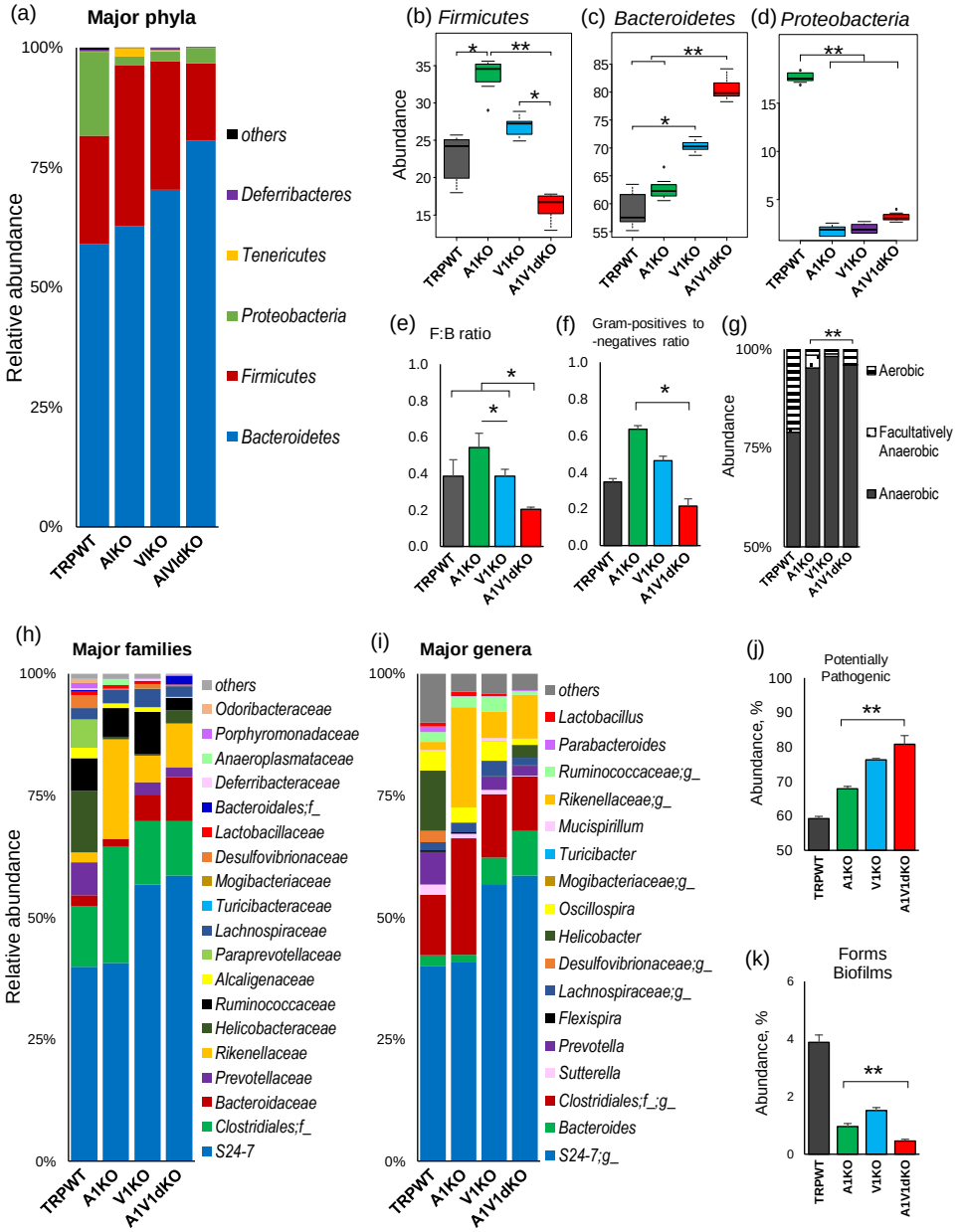

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Figure 2. Gut microbiome composition differs in mouse models of TRPA1 knockout, TRPV1 knockout and TRPA1/V1 double-knockout as compared to each other as well as to the TRP wild-type counterparts. (a) Gut microbiome composition at bacterial phylum level; (b-d) the abundance of major phyla; (e) ratio of Firmicutes to Bacteroides and (f) ratio of Gram-positive to Gram-negative taxa; (g) ratio of anaerobic to aerobic bacetria; and (h-i) microbiome composition at the level of major bacterial families (h) and genera (i); and (j-k) proportion of potentially pathogenic bacteria (j) and biofilm forming bacteria (k), in the mouse models of TRPA1 KO, TRPV1 KO and TRPA1/V1 dKO versus TRP-WT counterparts. *p<0.05, **p<0.001.

The analysis of relative abundance of major (top 15) bacterial taxa by hierarchal clustering clearly assort the whole cohort into four distinct clusters driven by the type of the KO (Fig. 3a), wherein A1 KO clusters close to the WT group whereas V1 KO and A1/V1dKO groups are clustered as separate distinct clusters. Similar clustering is demonstrated by the further analysis of Log_2-fold difference in the relative abundance of these bacterial taxa in KO vs. WT mice wherein V1 KO and A1/V1 dKO are clustered together and distinctly from A1 KO group (Fig. 3b). A1 KO cluster is characterized mainly by the markedly lower abundance of *Prevotella, Desulfovibrio, Bacteroides,* and *Helicobacter* and higher proportion of *Rikenellaceae* and *Tenericutes*; V1 KO mice demonstrate relatively higher abundance of *Ruminococcaceae, Lachnospiraceae, Ruminococcus, Desulfovibrio*, and *Mucispirillum*; whereas A1/V1 dKO groups is characterized by higher proportion of members belonging to the taxa *Bacteroidetes, Bacteroides* and *S24-7* and lower abundance of *Firmicutes, Ruminococcaceae, Oscillospira, Lactobacillus* and *Sutterella* (Fig. 3b). Further analysis of major bacterial taxa reveal several bacteria that are lower (Fig. 3c) or higher (Fig. 3d) in all the three knockout groups (although at different magnitudes) compared to the WT mice. Bacteria belonging to phyla *Proteobacteria* and *Cyanobacteria*, and families *Prevotellaceae*, *Helicobacteriaceae*, *Porphyromonadaceae* and *Desulfovibrionaceae* are lower in all the KOs mice whereas the population of phylum *Bacteroidetes*, and families *S24-7*, *Rikenellaceae* and *Mogibacteriaceae* is increased in all the three KOs compared to WT mice (Fig. 3c-d).

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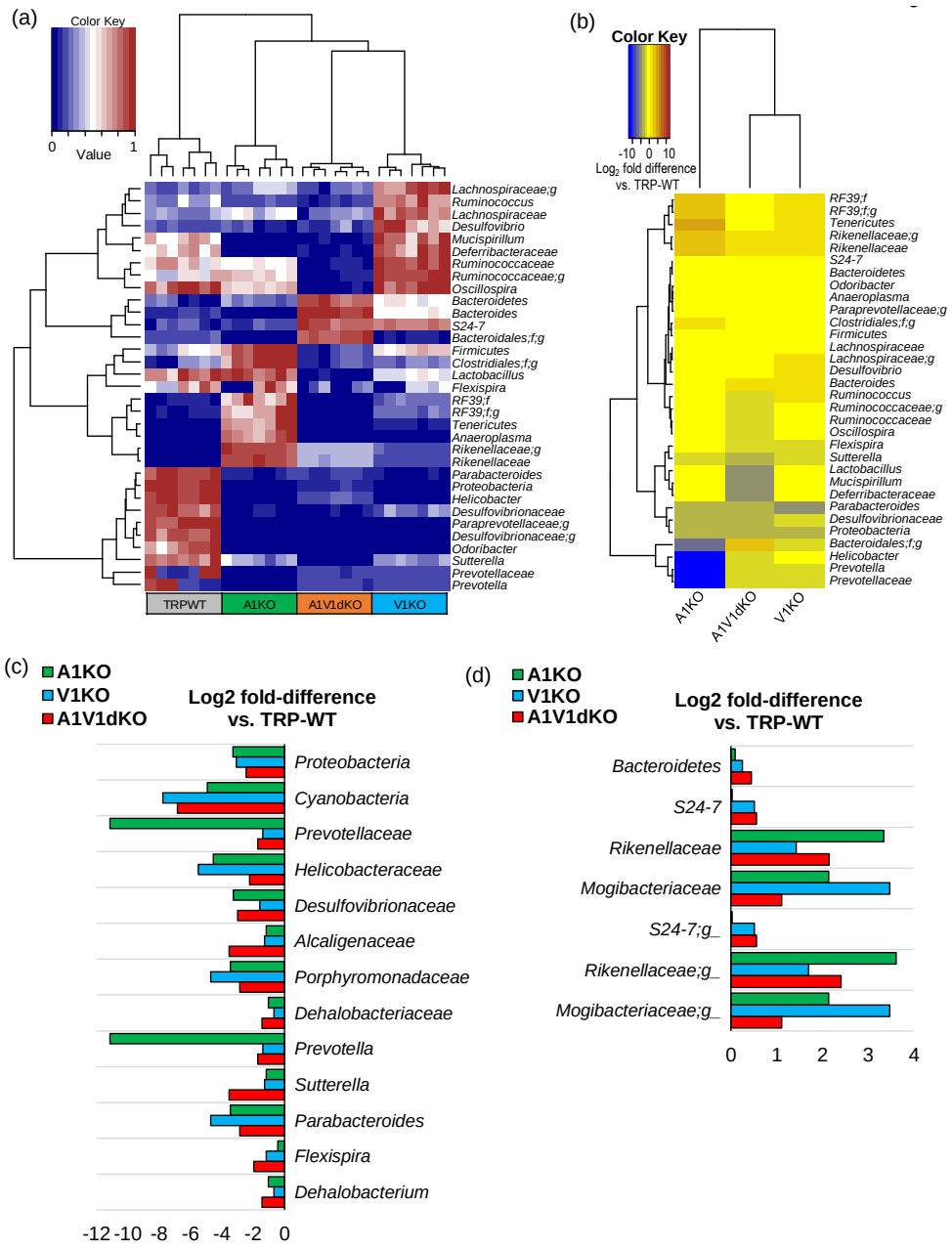

Figure 3. Differences in the abundance of various gut bacteria in mouse models of TRPA1 knockout, TRPV1 knockout and TRPA1V1 double-knockout as compared to each other as well as to the TRP wild-type counterparts. (a-b) Hierarchical clustering heap-map depicting distinct arrays characterized by different abundance levels (a) and Log_2 fold-differences (b) of major gut bacterial phyla, families and genera in mouse models of TRPA1 KO, TRPV1 KO and TRPA1/V1 dKO versus TRP-WT counterparts. (c-d) Major gut bacterial phyla, families and genera that were found to be reduced (c) or increased (d) in all of the three mouse models i.e., A1 KO, V1KO and A1V1dKO versus TRP-WT counterparts.

2.3. Mice with TRP-A1 KO, -V1 KO and -A1/V1 dKO genotypes present unique gut microbiome signatures

We then perform LEfSe (Linear discriminatory analysis [LDA] Effect Size) analysis to identify bacterial taxa that are unique in each group (Fig. 4a-b). As demonstrated by the LEfSe- generated cladogram (Fig. 4a) as well as in terms of the LDA score (Fig. 4b), the A1KO mice harbor higher proportion of *Firmicutes, Clostridia, Rikenella, Mollicutes* and *Lactobacillus* whereas V1KO mice microbiomes are enriched with members of *Clostridiales, Ruminococci, Lachnospira, Mogibacteria, Deferribacteriaceae*, and *Erysipelotrichaceae*. The A1V1dKO group is characterized by higher proportion

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of the members of the phylum *Bacteroidetes* including the representative genus *Bacteroides* as well as the next representative family *S24_7*. Further, all the three knockout groups have lower population of *Proteobacteria, Helicobacter, Prevotella, Sutteralla, Parabacteroides, Dehalobacterium* and *Cyanobacteria* as compared to the WT mice.

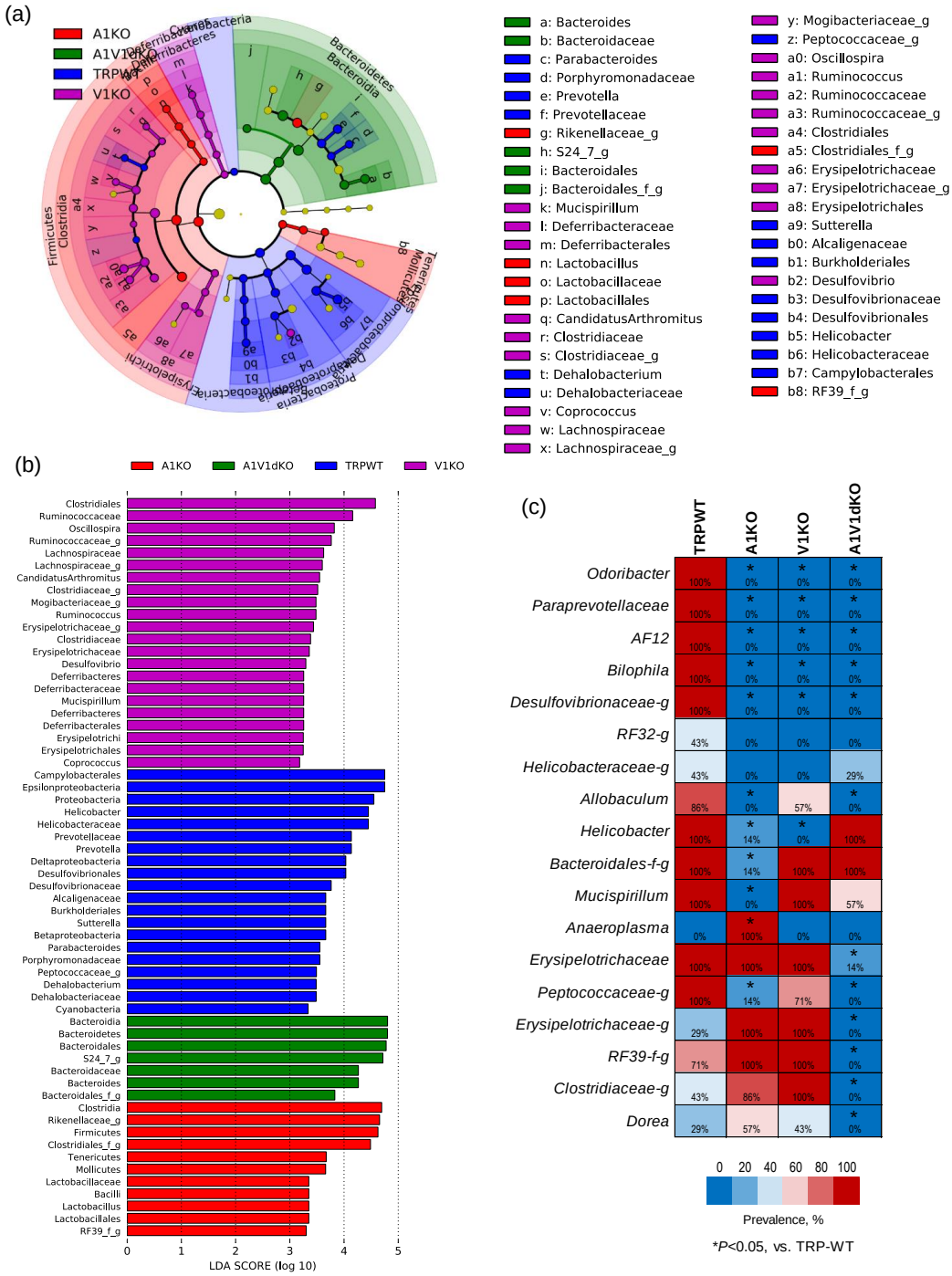

Figure 4. Unique gut microbiome signatures associated with specific mouse models of TRPA1 knockout, TRPV1 knockout and TRPA1V1 double-knockout. Linear Discriminatory Analysis (LDA) Effect Size (LEfSe) plot (a) and cladogram (b) showing bacterial taxa that are unique in mouse models of TRPA1 KO, TRPV1 KO and TRPA1/V1 dKO as well as in TRP-WT counterparts. (c) Differences in the detection rates of major bacterial phyla, families and genera in mouse models of TRPA1 KO, TRPV1 KO and TRPA1/V1 dKO versus TRP-WT counterparts.

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In addition to these uniquely abundant bacterial signatures, we also find several taxa that are unique in terms of their detection rate in the feces of mice with different TRP genotypes (Fig. 4c). As shown in the form of a heat-map in Fig. 4c, OTUs belonging to the bacterial taxa *Odoribacter, Paraprevotella, AF12, Bilophila* and *Desulfovibrionaceae* are detected in all the WT mice (detection rate: 100%) but remain undetected (detection rate: 0%) in all of the three KO mice, thereby indicating their association with the host TRP-genotype status. In addition, OTUs belonging to taxa *Peptococcaceae, Erysipelotrichaceae, RF39, Clostridiaceae* and genus *Dorea* are vanished in A1/V1 dKO mice but not in -A1 or -V1 KO mice. The genus *Anaeroplasma* is detected in A1 KO mice but remain completely undetected in WT as well as in V1 and A1/V1 dKO mice. In addition, the detection rate of *Mucispirillum* and an unclassified family of *Bacteroidales* is significantly lower only in A1 KO mice but not in V1 KO or A1/V1 dKO mice. On the other hand, the genus *Allobacullum* remains undetected in A1 KO and A1/V1 dKO mice but is detected in 57% of V1 KO mice. This indicates that TRP-gene deletion not only change the overall microbiome signature, but also represent unique microbial signature for each genotype.

2.4 Microbiome-related metabolic functions involved in the biosynthesis and metabolism of lipid and fatty acids are distinctly modulated upon deletion of specific TRP channels

The analysis of PICRUSt-inferred functional categorization of the gut microbiome followed by the Clusters of Orthologous Groups (COG) classification revealed many metagenomic functions related to the biosynthesis or metabolism of various lipids, fatty acids, carbohydrates, amino acids, vitamins, and other co-factors that were found to be significantly different between the different host genotypes. Of all these unique predicted functions (Suppl. Fig. 3), the abundance of KEGG pathways particularly associated with the biosynthesis or metabolism of lipid and fatty acids were found to be unique in each of the three KO mouse models (Fig. 5a). Interestingly, the abundance of gene families associated with the biosynthesis of lipids as well as the primary and secondary bile acids was found to be significantly higher in all of the three KO mice versus the WT mice. In contrast, the abundance of OTUs associated with overall fatty acid biosynthesis was found to be significantly lower in all these three TRP-KO groups. Whereas, the abundance of fatty acid metabolism-related families was significantly higher only in A1- and V1- KOs but not in dKO mice. The abundance of glycerophospholipid metabolism related OTUs was significantly higher in A1 KO mice as compared to WT as well as to V1 KO and TRPA1/V1 dKO mice. In addition, the abundance of OTUs associated with linoleic acid metabolism was significantly higher in V1 KO and -A1/V1dKO but not in TRPA1 KO mice. Further analysis of direct correlation of the abundance of these functional orthologs with the abundance of bacterial taxa revealed distinct array of correlation of specific bacteria with the biosynthesis or metabolism of lipids and fatty acids (Fig. 5b). OTUs belonging to the taxa *Enterococcaceae-RF39, Tenericutes, Anaeroplasma, Clostridiales, Firmicutes, Coprococcus, Ruminococcaceae, Erysipelotrichaeae, Aldercreutzia, Lactobacillus,* and *Dorea* were correlated negatively with the metagenomic functions related to lipids and fatty acids metabolism but positively with those related to biosynthesis. In contrast, OTUs belonging to bacterial taxa *Bacteroidetes, S24_7, Bacteroides* and *Prevotella* correlated positively with biosynthesis but negatively with the metabolism of lipids and fatty acids (Fig. 5b). The data indicate that TRPA1, TRPV1 and TRPA1/V1 deletion developed unique microbiome signatures that perform unique metabolic functions for modulation of lipid and bile acid metabolisms.

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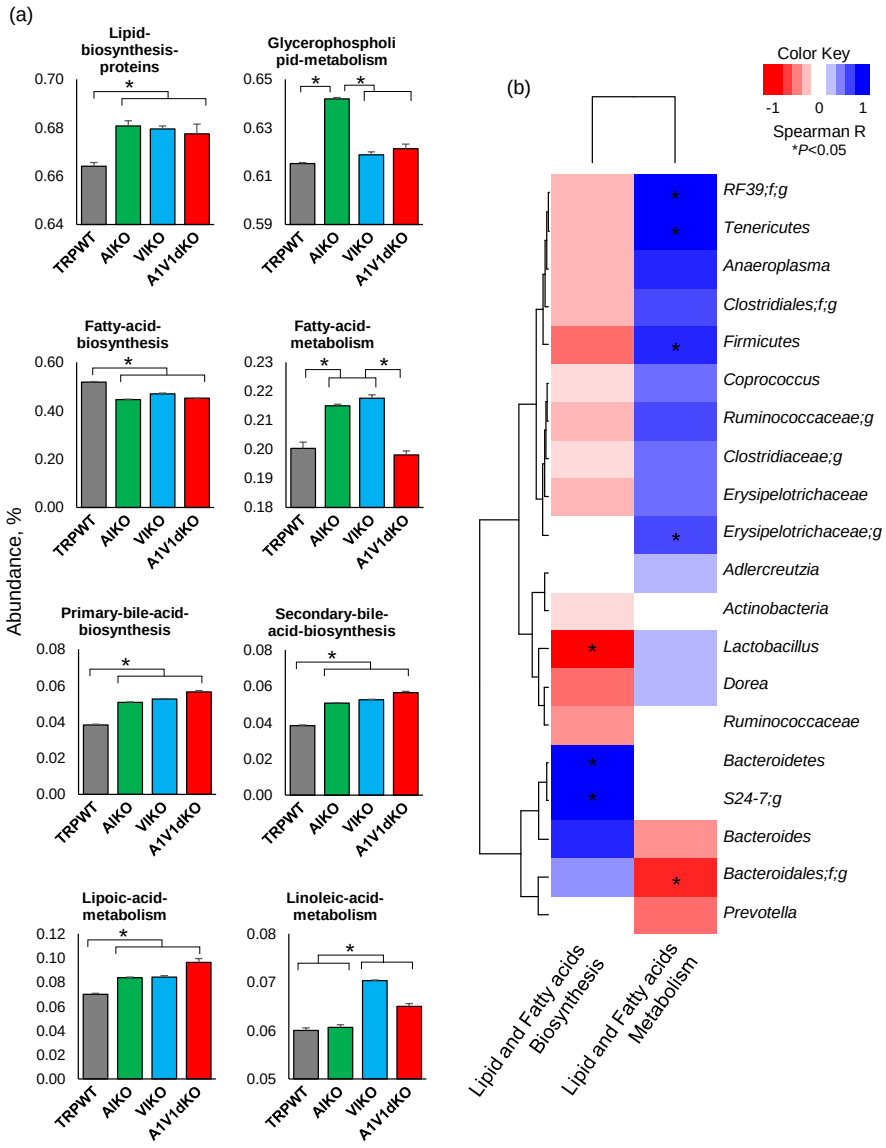

Figure 5. Differences in the functional analysis of the gut microbiome in mouse models of TRPA1 knockout, TRPV1 knockout and TRPA1V1 double-knockout. (a) The abundance of the predicted gut microbial metagenomic functions related to the lipids and fatty acids biosynthesis and metabolism pathways (Level 3 KEGG pathway) in mouse models of TRPA1 KO, TRPV1 KO and TRPA1/V1 dKO as well as in TRP-WT counterparts. (b) The hierarchical heat-map depicting the correlation of specific gut bacterial phyla, families and genera with functional metagenomic KEGG pathways related to the biosynthesis and metabolism of lipids and fatty acids.

3. Discussion

In this study, we examine the gut microbiome diversity and composition in TRPA1, V1, and A1/V1 knockout mice in comparison to their wild-type counterparts and identify distinct sets of gut bacterial signatures associated with the knockout of specific TRP genes involved in the pathophysiology of pain.

The gut microbiome analysis clearly reveal four distinct clusters (Fig. 1a) specific for four separate groups of mice viz. TRPA1 KO, -V1 KO, -A1/V1 dKO and TRP wild-type mice, thereby indicating different microbiome signatures between these four groups of mice. In addition, the α-diversity indices demonstrate a patterned decline in the bacterial diversity in the KO vs. WT groups (Fig. 1b) with diversity being modestly reduced in A1KO followed by considerably reduced in V1 KO while being remarkably reduced in the A1/V1dKO mice. Interestingly, both α- and β-diversity analyses show V1 KO and A1/V1 dKO mice to be closer to each other in comparison to TRP-WT and

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A1KO, indicating that both A1 and V1 knockouts may induce very distinct magnitude of impact on the gut microbiota diversity and composition. Notably, this decline in the microbial diversity concurs with several studies reporting reduced microbiome diversity in several pain-related disorders, such as fibromyalgia and myalgic encephalomyelitis, chronic fatigue syndrome[19,23,24]. Interestingly, our further analyses in KO groups demonstrate a reduction in the abundance of several bacterial taxa typically associated, as commensals, with a typical murine microbiome, such as *Prevotella*, *Helicobacter*, *Desulfovibrio*, *Sutterella*, *Parabacteroides*, and *Dehalobacterium* (Fig. 3c). These difference can be seen even at the highest level of taxonomic classification i.e., at phylum level that shows a clear significant reduction in *Proteobacteria* in unison with an expansion in the abundance of *Bacteroidetes* (Fig. 2a-d), thereby suggesting a dysbiotic (abnormal) microbiome spectrum in TRP KO vs. WT mice. Since there is no consensus as such at present on the use of the term "dysbiosis" or its meaning, it may be noted that the term "dysbiosis' in here is simply used to refer to a different gut microbiome composition in experimental groups versus wild-type (without any inference to whether it is casually or causally associated with the genetic manipulation or a disease manifestation).

Dysbiosis of the gut microbiome is also commonly reported to be associated with an abnormal intestinal epithelial permeability ('leaky gut') which may abnormally increase the interaction of gut bacteria with the host intestinal immune system (e.g., gut-associated lymphoid tissues) as well as the enteric nervous system (enteric neurons, neurotransmitters, etc.) eventually leading to increased episodes of local inflammation[32]. Such dysbiotic events have been previously reported in patients with chronic abdominal pain and also in patients with chronic widespread pain such as fibromyalgia[23,24]. In our TRP KO models, we find a decrease in the abundance of several members of the *Prevotellaceae*, *Paraprevotellaceae*, *Desulfovibrionaceae*, *Helicobacteriaceae* and *Clostridiaceae* and an increase in the proportion of *Bacteroidetes*, *Rikenellaceae* and *Mogibacteriaceae*. Such differences in the intestinal carriage of gut commensal along with a reduced overall diversity of bacteria, many of which are gut commensals (as seen in the wild-type counterparts) and are also involved in the nutrient digestion and the production of beneficial SCFAs, might also hint that such spectrum of gut dysbiosis might also be implicated in the comorbidities associated with the pathophysiology of pain. Hence, further studies undertaking a more inclusive and particularly the longitudinal investigation (lack of which is a limitation of our small study) is warranted to corroborate these findings as well as to gain deeper understanding of this otherwise under-explored microbiome-pain link.

The role of lipids and fatty acids in the propagation of pain is known to be like two opposite sides of the coin, which can either increase the pain sensitivity by activating the nociceptors present on the sensory neurons[33-36] or attenuate pain which is mediated by interaction with TRP channels[37-39]. Lipid mediators have previously been demonstrated to increase the pain sensitivity by activating G-protein coupled receptors (GPCR) that are linked through TRP channels acting downstream of the GPCR[40]. Anti-nociceptive effect of resolvins, the specialized proresolving lipid mediators, has been demonstrated to be due to the resolution of inflammation, which in turn facilitates reduction in pain sensitivity[41]. In addition, resolvins are the first endogenous inhibitors of TRPA1 and TRPV1 receptors that reduce pain by shutting the activity of these receptors by G-protein regulated mechanism[42]. During inflammation, the levels of resolvins are increased significantly in our body, although the potential mechanism(s) involved in this significant increase remains unknown. Despite the fact that these lipid mediators have two opposite effects on pain, it might be plausible that what regulates their synthesis and metabolism might be potentially associated with the gut microbiome. TRP channels act as an internal and external sensor or gate-keeper to regulate the gut microbiome, which in turn modulate pain sensitivity by regulating its synthesis and metabolism. Most importantly, our data on changes in gut bacteria that are involved in lipid biosynthesis and metabolism in TRP-deficient mice will provide some important clues about the role of these microbiota in nociceptive and anti-nociceptive processing. Taken together, the exact role of these gut microbial groups needs further exploration.

The results reported here are, to the best of our knowledge, the first to demonstrate gut microbiome differences in TRPA1 and V1 knockout as well as A1/V1 dKO mice in comparison to their wild-type counterparts. Some of the bacterial taxa reported here have previously been known

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to be involved in several host metabolic pathways whose association with the neuronal channels involved in pain might be biologically plausible. Moreover, there appears to be a somewhat quantitative association between the abundance of several taxa and the type of TRP channel knockout, hinting at the potential connection between TRP channel proteins and the gut microbiome. Pertaining to the limitations of the present study, it may be noted that our data are limited to the results obtained from the high-throughput sequencing of the bacterial 16S rRNA gene only. Further studies employing the whole bacterial metagenomic sequencing (e.g., shot-gun sequencing approaches) and also the metatranscriptomics and metabolomics tools would not only be able to identify more and novel microbial and metabolic biomarkers but may also upgrade the characterization of potentially implicated biological mechanisms and pathways underlying the pain pathophysiology. It should also be noted that the findings reported in here are from although well-established but still rodent models. Hence, it is difficult to be certain whether and how the detected microbiome differences in these TRP KO mice would extrapolate or translate to the human milieus. Furthermore, human subjects suffering from impaired pain perception or pain-related disorders are also highly likely to have a different dietary and lifestyle routines and undertake different therapeutic regimens, all of which could influence their gut microbiota assemblage. Further and more comprehensive studies examining the possible microbiome changes in other similar genotypes and phenotypes of pain and exploring the potential causal versus casual association between the gut microbiome and pain physiology should endeavor to validate and further comprehend this otherwise under-explored microbiome-pain axis. Nevertheless, the data offer avenues to broaden our understanding of the pathophysiology of pain and perhaps could also facilitate outlining the future diagnostic and therapeutic modalities. Exploration of mechanisms by which the gut microbiome and microbial metabolites may affect the functions of TRP receptors could offer novel insights into the pathophysiology of pain and might also possibly lead to the use of microbiome data in the diagnosis of pain in the future. Indeed, if a causal relationship between gut microbiome and the pathophysiology of pain is established, the way may be paved for the development of novel diagnostic and treatment strategies exploiting this intriguing community of gut microbes. Hence, our data should help to shed some new light on the pathophysiology of pain in particular context to the intestinal microbial ecosystem and may help reveal novel markers within a biological and physiological framework while improving our understanding of this relatively unknown phenomena of TRP-related pain sensation.

4. Materials and Methods

Animals. All experiments and procedures were performed in accordance with the North Carolina State University laboratory animal care. TRPs knockout mice were generated per our previously described methods[43,44] and were maintained on the inbred strain with identical strain-matched wild-type littermates. The mice (male; n=7 per group) were 10-12 week-old at the time of mechanical pain measurements and the fecal collection. Validation of knockout was assessed by measuring mechanical pain using a von-Frey apparatus (Ugo Basile), as described in our previous study[44].

Gut microbiome analysis. Gut microbiome was examined as per our previously described methods[45-49]. Briefly, bacterial genomic DNA from fecal specimens was extracted by using Qiagen DNA Stool Mini Kit (Qiagen, Valencia, CA, United States) with slight modification described previously[45]. The V4 hypervariable region of the 16S rDNA gene was PCR amplified using the universal primers 515F (barcoded) and 806R; the resulting amplicons were cleaned up with AMPure® magnetic purification beads (Agencourt); the purified products were quantified using the Qubit-3 fluorimeter (InVitrogen); and the amplicon library was generated according to methods described elsewhere[50]. The purified PCR product was pooled in equal molar concentrations and sequenced on an Illumina MiSeq platform using 2x300bp reagent kit (MiSeq reagent kit v3; Illumina Inc., CA, USA) for paired-end sequencing. The obtained sequences generated were de-multiplexed, quality-filtered, clustered, and taxonomically assigned against Greengenes database with RDP-classifier using QIIME (Quantitative Insights into Microbial Ecology) software package[51] as described previously [46-48]. To avoid bias due to different sequencing depth, the sequences were rarefied to the lowest number of sequences per sample for downstream analyses. Alpha-dievrsity indices were

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computed within QIIME. Beta diversity was analyzed using principal coordinate analysis (PCoA) of the unweighted and weighted Unifrac distance (using EMPeror version 0.9.3-dev). Bacterial taxonomy assignment was calculated within QIIME using default settings to compare the bacterial diversity and abundance between the different groups. The proportions of microbial organism-level phenotypes were computed using open-source algorithm BugBase. The metabolic and other functional activities were computed using the open source bioinformatics tool PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States)[52]. The sequences were uploaded to PICRUSt and were analyzed for the prediction of functional genes of the classified members of the gut microbiota against Greengenes database. Subsequently, the inferred gene families were annotated against KEGG (Kyoto encyclopedia of genes and genomes) orthologs (Kos) and then collapsed into KEGG pathways to generate the functional pathway. The functions were finally categorized and compared at levels 2 and 3 as per the methods described elsewhere[52].

Statistical analyses. α-diversity indices and bacterial abundance between TRP wild-type and KO mice were compared using Kruskal-Wallis test followed by Dunn's post-hoc multiple pairwise comparison test. Hierarchical clustering and heat-maps depicting the patterns of abundance and log values were constructed R statistical software package (version 3.6.0; https://www.r-project.org/) using the 'heatmap.2' and "ggplots" packages. LEfSe (Linear discriminatory analysis [LDA] Effect Size)[53] was used to identify discriminative features (unique bacterial taxa) that drive differences in KO vs. WT mice. Differences in β-diversity were tested by permutational multivariate analysis of variance, a permutation-based multivariate analysis of variance to a matrix of pairwise distance to partition the inter-group and intra-group distance. Correlation between bacterial abundance and the index of pain measurement was estimated by Spearman's rank correlation coefficient test (GraphPad Prism software system, version 6.0). In all experiments, significance threshold was set at $p < 0.05$. Unless otherwise stated, all the values presented herein are means ± SEM.

Supplementary Materials: Supplementary figures have been provided along with this manuscript. All the raw sequencing data sets have been submitted to the NCBI Sequence Read Archive database under SRA accession number: SUB6718095 and bio-project number PRJNA597161.

Author Contributions: SM, HY: conceived and designed research; RN: analyzed microbiome; wrote manuscript; SM: overlooked mice maintenance and performed experiments; SM, HY, GD: interpreted results of experiments and data analyses; HY, SM, GD: revised and edited manuscript; RN, HY, SM, GD: approved final version of manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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