Gut microbiota promotes enteroendocrine cell maturation and mitochondrial function

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Abstract

The enteroendocrine cells (EECs) in the intestine are crucial for sensing ingested nutrients and regulating feeding behavior. The means by which gut microbiota regulates the nutrient-sensing EEC activity is unclear. Our transcriptomic analysis of the EECs from germ-free (GF) and conventionalized (CV) zebrafish revealed that commensal microbiota colonization significantly increased the expression of many genes that are associated with mitochondrial function. Using in vivo imaging and 3D automated cell tracking approach, we developed new methods to image and analyze the EECs' cytoplasmic and mitochondrial calcium activity at cellular resolution in live zebrafish. Our data revealed that during the development, shortly after gut microbiota colonization, EECs briefly increased cytoplasm and mitochondrial Ca^{2+}, a phenomenon we referred to as "EEC awakening". Following the EEC awakening, cytoplasmic Ca^{2+} levels but not mitochondrial Ca^{2+} level in the EECs decreased, resulting in a consistent increase in the mitochondrial-to-cytoplasmic Ca^{2+} ratio. The increased mitochondrial-to-cytoplasmic Ca^{2+} ratio is associated with the EEC maturation process. In immature EECs, we further discovered that their mitochondria are evenly distributed in the cytoplasm. When EECs mature, their mitochondria are highly localized in the basal lateral membrane where EEC vesicle secretion occurs. Furthermore, CV EECs, but not GF EECs, exhibit spontaneous low-amplitude calcium fluctuation. The mitochondrial-to-cytoplasm Ca^{2+} ratio is significantly higher in CV EECs. When stimulating the CV zebrafish with nutrients like fatty acids, the fatty acid responsive EECs' cytoplasmic and mitochondrial calcium levels both increase. Strikingly, when applied to GF zebrafish, the nutrient stimulation increases cytoplasmic Ca^{2+} levels, but the nutrient stimulation induced mitochondrial Ca^{2+} increase that was observed in CV zebrafish was now nearly abolished. Together, our study reveals that commensal microbiota are critical in supporting EEC mitochondrial function and maturation. Selectively manipulating gut microbial signals to alter EEC mitochondrial function will provide new opportunities to change gut-brain nutrient sensing efficiency and feeding behavior.

Introduction

Feeding behavior is conserved among all organisms. During development, the fetus receives its nutrient supply from its mother. Immediately after birth, the maternal nutrient supply is cut off and the infant needs to initiate the feeding process to obtain nutrients. After eating, the ingested nutrients need to be sensed, and such nutrient information will

be transmitted to the rest of the body to coordinate its metabolic function [1]. Within the intestinal epithelium, a group of specialized sensory cells called enteroendocrine cells (EECs) sense the ingested nutrient information and secrete hormone molecules to regulate physiological homeostasis [2]. The EECs are critical nutrient sensors in the intestinal epithelium. They are dispersed along the digestive tract and comprised of less than 1% of the intestinal epithelium cells. However, collectively, the EECs form the largest endocrine organ in the body [2]. Most of the previous studies assessing EECs are focused on adults. It is well-known that ingested nutrients, such as fatty acids or glucose, directly stimulate the EECs by triggering a cascade of membrane depolarization, action potential firing, voltage-dependent calcium entry, and hormone-containing vesicle release [2]. Many of these EEC-secreting hormones, like Cholecystokinin (CCK) or glucagon-like peptide 1 (GLP-1), are critical in regulating satiation response and metabolism [1, 2]. In addition to the classic hormone secretion function, recent research also demonstrated that the EECs form a basal membrane process called “neuropod” that directly synapses with the vagal sensory neurons [3, 4]. Through the EEC-vagal neuronal pathway, ingested nutrient information in the gut lumen can be transmitted to the brain [4]. Further studies demonstrated that this nutrient-sensing EEC-vagal pathway is essential to drive animal’s food preference toward sugar and fat [5-7]. It is well known that the intestinal epithelium cells undergo significant remodeling during the postnatal period to adapt to the need for nutrient absorption and sensation [8]. Despite the importance of EECs in nutrient monitoring, gut-brain nutrient sensing, feeding behavior, and systemic metabolic regulation, little is known about how environmental factors change EEC maturation and function during the postnatal developmental period.

Following birth, newborn babies are rapidly colonized by microbial organisms [9]. These microbial organisms start to assemble the functional microbial community that plays important roles in the development of the infant [9]. Previous studies revealed that microbiota colonization during the early postnatal phase is critical in promoting intestinal epithelium maturation and remodeling [10-12]. Numerous pieces of evidence also suggest that gut microbiota are important in regulating nutrient absorption, metabolism, and infant growth [13-15]. Research from animal models and clinical studies suggest that gut microbiota are critical in modulating feeding behavior, including appetite and food choice [16]. However, little is known about how gut microbiota interacts with EECs and regulates the EECs' function during development. Whether and how gut microbiota interacts with EECs to modulate postnatal physiological homeostasis remains unknown.

Mitochondria is the essential organelle that provides ATP to sustain cellular function. The mitochondria emerge as a key player in coordinating cellular metabolism, cell differentiation, and regulating intestinal epithelium homeostasis [17-19]. In the nervous system, mitochondria are important in supporting neuronal vesicle secretion, neuronal synaptic transmission, and neuronal network formation [20]. Research in pancreatic endocrine cells also revealed that mitochondrial function is essential for pancreatic beta-cell hormonal secretion, and mitochondrial activity is inhibited in diabetic conditions [21, 22]. How EEC mitochondrial activity changes during development remains unknown. Little is known about the physiological roles of the mitochondrial function in EECs. How environmental factors regulate EEC mitochondrial activity in vivo is unknown.

A major challenge in studying how environmental factors, such as gut microbiota, regulate EEC physiology has been the lack of in vivo techniques to study this rare intestinal epithelium population in the intact animal setting. Historically, these cells have been studied by measuring the circulating EEC-secreting hormones [2]. However, many EEC hormones have very short half-lives and the plasma hormone level does not mirror the EEC function nor the real-time EEC activity [23]. EEC activity has also been studied via cell culture or organoid culture systems. However, a cell or organoid culture is not able to mimic the dynamic and complex intestinal luminal factors that shape the EECs. It is also difficult to study how EECs communicate with neighboring cells or distant organs, like the brain, using the in vitro culture system.

In this study, we utilized the zebrafish model to examine how commensal microbiota affect EEC maturation and function. Using an innovative approach to direct images and track the EEC cellular and mitochondrial calcium activity in live zebrafish during development, our results revealed that the EEC morphology, cellular, and mitochondrial activity is dynamically regulated during the EEC maturation process. Importantly, our results revealed that gut microbiota play critical roles in promoting EEC maturation and mitochondrial function.

Results

Gut microbiota alters EEC subtypes

Previous studies including ours demonstrated that similar to mammals, the zebrafish EECs have diverse subtypes [24, 25]. A recent zebrafish intestine epithelium single-cell RNA sequencing dataset further revealed the five EEC subtypes in the zebrafish larvae characterized by their distinct hormone expression profiles (Fig. S1A-B) [26]. EEC1 is characterized by the expression of the hormonal genes: pancreatic polypeptide b (*pyyb*), somatostatin 2 (*sst2*), and ghrelin (*ghrl*) (Fig. S1A-B). EEC2 expresses the hormonal genes: preproglucagaon a (*gcga*), the gene that encodes Glucagon (Gcg) or Glucan-like-pepetide 1 (GLP-1), vasoactive intestinal polypeptide b (*vipb*), and insulin-like 5 (*insl5*) (Fig. S1A-B). EEC3 expresses the hormonal genes: calcitonin related polypeptide alpha (*calca*) and neuromedin b (*nmb*) (Fig. S1A-B). EEC4 expresses the hormonal genes: cholecystokinin a (*ccka*) (Fig. S1A-B). EEC5 uniquely expresses the following hormonal genes: brain-derived neurotrophic factor (*bdnf*), adenylate cyclase-activating peptide-1 a (*adcyap1a*), preproenkephalin a (*penka*), and tryptophan hydroxylase 1 b (*tph1b*), the enzyme that synthesizes serotonin (Fig. S1A-B). The EEC5 also highly and uniquely expresses the Transient receptor potential ankyrin 1 b (Trpa1b). These Trpa1+EECs that are characterized in our previous studies sense microbial stimulants and are critical in regulating gut motility and intestinal homeostasis [27]. Some of these EEC markers can be labeled via immunofluorescence staining and transgenic approaches (Fig.S1C-K). Our results confirmed the zebrafish EEC subtype hormonal profile revealed in the single-cell RNA sequencing above (Fig. S1C-K). Moreover, consistent with previous studies, our data revealed that the distribution of the EEC subtypes exhibits regional specificity (Fig. S1C-K). For example, the PYY+EECs are exclusively in the proximal intestine, while the Trpa1+EECs are distributed along the whole digestive tract. Interestingly, the Trpa1+EECs (EEC5) appeared to have heterogeneity. As the proximal intestinal

Trpa1+EECs express both Enk and Serotonin (Fig. S1C-K). However, the middle and distal intestinal Trpa1+EECs do not express ENK but only express serotonin (Fig. S1C-K).

Next, we investigated how commensal gut microbiota affect EEC subtype specification via the zebrafish gnotobiotic approach (Fig. 1A) [28]. The zebrafish were derived as germ-free at 0dpf (days post fertilization) [28]. At 3dpf, the zebrafish are maintained as germ-free (GF) or colonized with commensal microbiota (Conventionalized, CV). The gnotobiotic zebrafish were fed from 3dpf to 7dpf, and the zebrafish were fixed at 7dpf for immunofluorescence staining. Our results revealed that commensal microbiota colonization did not significantly alter the percentage of PYY+ EECs and ENK+EECs in the proximal intestine (Fig. 1B-E', J-K). However, the GF zebrafish increased the number of Gcg/GLP-1+EECs in the proximal intestine and Trpa1+EECs in the distal intestine (Fig. 1F-I, L-N).

Gut microbiota promotes EEC maturation and mitochondrial function

To further understand how commensal microbiota modulate EECs in zebrafish, we used Flow Activated Cell Sorting (FACS) to isolate EECs from GF and CV zebrafish. Then, we performed transcriptomic analysis (Fig. 2A). For each gene, we plotted the fold change in response to gut microbial status (CV vs GF) and fold change in response to the cell fate (EEC vs other IEC) (Fig. 2B). Our results demonstrated that there is a weak but significant positive correlation between genes that are enriched in EECs and the genes that are upregulated in the CV condition(Fig. 2B). Within the genes that are significantly upregulated in CV, about 74.5% of them are enriched in EECs (Fig. 2C). We then plotted the conserved EECs' signature genes that are shared among zebrafish, mice, and humans [27]. Our results indicate that about 75% of the conserved EEC signature genes are upregulated in CV (Fig. 2D). Within those conserved EEC genes, many of them are associated with EEC cell membrane potential regulation and vesicle secretion (Fig. 2E). The CV condition also significantly upregulates the chromogranin A gene (*chga*), which labels the mature EECs (Fig. 2E). Therefore, consistent with the immunofluorescence results above, the transcriptomic analysis indicates that gut microbiota promote EEC cell fate and maturation. Next, we performed Go Term analysis of the genes that are significantly upregulated in CV and genes that are significantly upregulated in GF using the Metascape gene analysis tool (Fig. 2F-G). Interestingly, in the genes that are significantly upregulated in GF EECs, top Go Term includes gene functions related to adhesion, migration, and actin filament-based processes (Fig. 2G). Within the CV upregulated genes, several Go Terms associated with mitochondrial function are enriched (Fig. 2G).

Next, we annotated all of the genes associated with different aspects of mitochondrial function (Table). We found that almost all of the genes in the FASTK mitochondrial RNA binding family, TIM23 complex, and mitochondrial contact site and cristae organizing system are upregulated in CV (Table). The mitochondrial DNA encodes 13 proteins that are critical for electron transport chain reactions [29]. The FASTK (FAS-activated serine/threonine kinase) gene family is located in the mitochondrial matrix and plays an important role in processing RNA transcribed from the mitochondrial DNA [30]. The

FASTK gene family is essential for synthesizing the components of the electron transport chain. Within the FASTK family, previous studies showed that FASTK and FASTKD2 increase the NADH dehydrogenase transcripts and promote mitochondrial respiration specifically [30, 31]. FASTKD2 is one of the most significant genes upregulated in CV EECs (Fig. 2H). Mitochondria acquire most of their protein from the cytosol [32]. The TIM23 complex is essential for translocating cytosolic preprotein into the mitochondrial matrix across the mitochondrial membrane [32]. Inside the mitochondria, the inner membrane forms invaginations known as cristae. The cristae are very specialized structures that support respiration [33]. Our results indicate that many genes that are associated with cristate organization are upregulated in CV EECs (Tabel), suggesting that commensal microbiota colonization increases EEC mitochondrial respiration function. In addition to the genetic pathways above, our results also indicate that cox10 (an important component of mitochondrial respiration for complex III) is among the most significantly upregulated CV EEC genes (Fig.2H). Together, our transcriptomic data indicates that gut microbiota promote EEC maturation and mitochondrial function by increasing different genetic pathways that are involved in mitochondrial respiration (Fig. 2I).

Immature EECs contain active filopodia structures at the basal lateral membrane.

Our RNA sequencing results revealed that GF zebrafish exhibit increased gene expression related to their actin filament. We then used a *Tg(neurod1:lifeActin-EGFP)* transgenic zebrafish model [24] to examine EEC actin filament dynamics. We examined both the 3dpf and 6dpf proximal intestines of the fixed zebrafish samples. The zebrafish EECs start to form at around 60 hours post fertilization. At around 3dpf, the zebrafish hatched from their chorion. The gut lumen opens and gut microbiota starts to colonize the intestine. Previous studies demonstrated that the intestinal epithelium cells, including EECs, are highly polarized and contain a dense actin network in the microvilli at the apical brush border [24]. To our surprise, our data showed that at 3dpf, almost all of the zebrafish EECs exhibit complex actin filament protrusions at the base (Fig. 3A-A'). Interestingly, we did not detect active basal actin filament in other IECs at 3dpf zebrafish (data not shown). This indicates that the formation of the basal actin filaments is not associated with the general intestinal epithelium development process. It is a unique phenomenon that involves immature EECs. By 6dpf, the EECs' basal actin filaments disappeared. Most EECs exhibited typical spindle-type morphology with a flat base (Fig. 3B-B'). To examine the EEC actin filament dynamic change further, we performed live imaging of the EECs at 3dpf *Tg(neurod1:lifeActin-EGFP)* in zebrafish and traced the same fish to 6dpf. Consistently, we observed that at 3dpf, almost all the EECs have complex actin filopodium filaments in the basal lateral portion, and the EEC extends and retracts their filopodium filaments constantly (Fig. 3C-D') (video). However, in the same zebrafish, by 6dpf, the EECs do not have actin filaments at the base but contain a high lifeActin-EGFP signal at the apical brush border (Fig. 3E-F') (video). Our data revealed for the first time that the immature EECs have an active filopodia process at the basal lateral membrane. When the EECs start to develop and mature, they become more polarized and lose their basal filopodia process. In general, filopodia are an antenna for cells to probe their environment. They are associated with the neuron axon growth cone and serve as precursors for dendritic spines. The function of the immature EEC filopodia requires future investigation.

To investigate how gut microbiota regulate the EEC filopodia process and maturation, we generated GF and CV *Tg(neurod1:lifeActin-EGFP)* zebrafish. We imaged their proximal intestine at 7dpf. Our results demonstrated that about 15% of the 7dpf GF zebrafish EECs in the proximal intestine still have active filopodia actin filament at the base (Fig. 3E,G). The ratio of the EECs with active filopodia actin filament is significantly reduced in CV zebrafish (Fig. 3F, G). This data suggests that certain microbial cues promote EEC maturation and facilitate the disappearance of the EEC filopodia actin filament. This data is also consistent with the RNA seq results above, regarding the GF zebrafish EECs' increased gene expression associated with the actin-filament based process (Fig. 1G).

Previous mice studies suggested that EECs form an extended membrane process called "neuropod" to connect with the nervous system. Interestingly, we observed that some EECs in 7dpf zebrafish intestines formed an extended membrane process in the basal membrane that morphologically resembles the mammalian neuropod of EECs (Fig. S2C-D'). The extended membrane process is distinct from the thin actin filopodia filament and is not detected in 3dpf zebrafish EECs. The neuropod-like EECs are rare in the GF zebrafish intestine (Fig. S2A-B'). The CV zebrafish exhibit a significantly higher percentage of neuropod-like EECs in the intestine (Fig. S2E). Our data suggests the formation of the neuropod-like structures in mature EECs which require certain microbial cues.

Commensal microbiota promotes the formation of mitochondria hot spots in the EEC basal membrane.

Next, we seek to determine how gut microbiota regulate the EECs' mitochondria. We used a *Tg(neurod1:mitoEOS)* transgenic zebrafish model to visualize the EECs' mitochondria [34]. In this model, the green fluorescent EOS protein contains a mitochondrial tag that is expressed in EECs to label their mitochondria. Using this model, we can trace the EEC mitochondrial abundance and intracellular mitochondrial distribution in live zebrafish over time. Our results revealed that, from 3dpf to 5dpf, the zebrafish EEC did not significantly increase the mitochondrial abundance (Fig. S3A). However, at 6dpf, EECs exhibit higher mitochondrial abundance compared with 3dpf-5dpf EECs (Fig. S3A). Interestingly, at 3dpf, the mitochondria are evenly distributed within the EECs, and the mitochondrial contents near the basal lateral membrane are low (Fig. 4A-C'). By 6dpf, most EECs exhibit hot spot mitochondrial distribution patterns (Fig. 4D-E'). High mitochondrial contents are found at the base of EECs, presumably at the sites where EECs secrete vesicles (Fig. 4D-E'). When we compare GF and CV zebrafish, commensal microbiota colonization did not alter the proximal intestinal EEC mitochondrial abundance but decreased the distal intestine EEC mitochondrial abundance (Fig. S3B-C). However, the CV zebrafish increases the percentage of EECs with high basal membrane mitochondrial contents (Fig. 4F-I).

Mature EECs increase mitochondrial activity

To analyze the dynamic change of EECs' cellular and mitochondrial activity during their development and maturation process, we used the *Tg(neurod1:Gcamp6f); Tg(neurod1:mito-RGECO)* dual transgenic model [34]. In this model, the green

fluorescent calcium indicator protein Gcamp6f is expressed in the EEC cytoplasm. A red fluorescent calcium indicator protein RGECO that contains a mitochondrial tag is expressed in the EEC mitochondrial matrix. Therefore, by using this dual transgenic model, we can simultaneously measure EEC cytoplasmic Ca^{2+} levels and mitochondrial Ca^{2+} levels by measuring the change in green and red fluorescence (Video). We then analyzed how EEC cytoplasmic and mitochondrial Ca^{2+} levels change during development by tracing the same zebrafish from 3dpf to 6dpf (Fig. 5A-E''). Our results showed that at 3dpf, the EECs exhibit low cytoplasmic and mitochondrial Ca^{2+} levels (Fig. 5B-B'', G, H). Interestingly, at 4dpf, there is a significant increase of both EEC cytoplasmic and mitochondrial Ca^{2+} levels (Fig. 5C-C'', G, H). From 5dpf to 6dpf, the EEC cytoplasmic Ca^{2+} levels decreased, while mitochondrial Ca^{2+} levels remained high (Fig. 5D-E'', G, H). As a result, from 3dpf to 6dpf, the EEC mitochondrial to cytoplasmic Ca^{2+} ratio continues to increase (Fig. 5F). A similar trend was found in all of the zebrafish samples that we traced (Fig. S5). When we grouped data of zebrafish of the same age together, we also observed that as the EECs became more mature, the EECs increased their mitochondrial Ca^{2+} levels and EEC mitochondrial to cytoplasmic Ca^{2+} level ratio (Fig. 5I-K).

Gut microbiota increases resting EEC mitochondrial activity and spontaneous firing

Our new genetic zebrafish model and imaging approaches allow us to investigate how gut microbiota changes EEC cellular and mitochondrial activity in vivo. We generated *Tg(neurod1:Gcamp6f); Tg(neurod1:mitoRECO)* GF and CV zebrafish. We imaged the fish's proximal intestinal EECs at 7dpf (Fig. 6A). First, we examined the absolute EEC cellular plasma Ca^{2+} levels and EEC mitochondrial Ca^{2+} levels by analyzing the individual EEC Gcamp6f and EEC mitoRGECO fluorescent levels in GF and CV zebrafish. Compared to GF EECs, CV EECs exhibit significantly lower cytoplasmic Ca^{2+} levels (Fig. 6A-C'') (Fig. S4). However, the CV EECs exhibit a significantly higher mitochondrial to cytoplasmic Ca^{2+} ratio (Fig. 6A-C'', Fig. 6D-F) (Fig. S4). This result suggests that gut microbiota may promote low resting EEC cytoplasmic Ca^{2+} levels but increase EEC mitochondrial activity. This finding is consistent with the results from our RNA sequencing analysis above (Fig.2).

Using a 3D cell tracking approach, we can automatically track individual EECs and analyze their Gcamp6f and mitoRGECO fluorescent change on a temporal scale. We can image and analyze the spatiotemporal EEC cellular and mitochondrial activity change in real time at the cellular resolution. We analyzed the relative EEC cytoplasmic Ca^{2+} and mitochondrial Ca^{2+} levels change in GF and CV zebrafish. For each EEC, we normalized the EEC Gcamp6f, EEC mitoRGECO, and EEC mitoRGECO/Gcamp6f ratio value to their values at time 0. Our results indicate that some EECs in the CV zebrafish exhibit low amplitude firing as reflected by the temporal fluctuation of the EEC cytoplasmic Ca^{2+} levels (Fig. 6K-M) (Video). However, this spontaneous firing is not apparent in the GF zebrafish EECs (Fig. 6G-I) (Video). Analysis of EECs across different GF and CV zebrafish samples indicate that ~21% of CV EECs exhibit low amplitude firing, but only 2.5% of GF EECs exhibit low amplitude firing (Fig. 6J, N). Those EECs with spontaneous firing increase the relative mitochondrial Ca^{2+} levels but not the relative mitochondria-to-cytoplasm Ca^{2+} ratio (Fig. 6H, L). These results indicate that in the spontaneously firing

EECs, mitochondria may not be the main source to buffer the cytoplasmic Ca^{2+} increase. These results suggest that at the resting condition, the CV EECs have higher mitochondrial activity and their mitochondrial activity is more dynamic.

Nutrient-induced EEC mitochondrial calcium increase requires gut microbiota

As the primary sensory cells, one of the major functions of EECs is to sense the intestinal lumen's nutrients. Using the *Tg(neurod1:Gcamp6f)* zebrafish model, we previously developed a method to image live zebrafish's EEC response to nutrients using an epifluorescent microscope [24]. Our previous methods allowed us to examine the systemic intestinal EEC nutrient response. However, it does not enable us to image the EECs' nutrient sensing function at the cellular level [24]. To analyze how EECs respond to nutrients at the cellular level in live zebrafish, we developed a method to give stimulants to the zebrafish when we perform confocal imaging. Using 3D segmentation and 3D objective tracing, we can then quantify individual EECs' response to nutrient stimulation systemically (Fig. 7A). Similar to mammals, the zebrafish EECs respond to nutrient stimulants like fatty acid [24, 35]. Previous studies suggest that long-chain fatty acids like linoleic acid bind with the G-protein coupled receptor in zebrafish EECs, stimulating calcium release from ER (Fig. 7B) [35]. Whether and how nutrient stimulation modulates EEC mitochondrial Ca^{2+} levels remains unclear.

Using calcium imaging, EEC tracking, and cluttering analysis, we observed three EEC subgroups that displayed different cytoplasmic and mitochondrial Ca^{2+} level responsive patterns post-linoleic acid stimulation: (1) Both EEC cytoplasmic Ca^{2+} and mitochondrial Ca^{2+} levels are activated by linoleic acid stimulation (Fig. 7C-J, K, O); (2) Only the EEC cytoplasmic Ca^{2+} levels, but not the mitochondrial Ca^{2+} levels, are activated by linoleic acid stimulation (Fig. 7L, O); (3) Neither the EECs cytoplasmic nor mitochondrial Ca^{2+} are activated by linoleic acid. (Fig. 7M, O). In the first EEC subgroup, after linoleic acid stimulation, they exhibited a significant increase in their cytoplasmic Ca^{2+} level (Fig. 7K). The cellular cytoplasmic Ca^{2+} levels in these cells quickly returned to their basal activity level (Fig. 7K). Their mitochondrial Ca^{2+} levels increased immediately following the cytoplasmic Ca^{2+} peak (Fig. 7K). However, unlike the cytoplasmic Ca^{2+}, the mitochondrial Ca^{2+} level remained higher than the basal Ca^{2+} level after the peak (Fig. 7K). As a result, the relative mitochondrial-to-cytoplasmic Ca^{2+} ratio increased post- linoleic acid stimulation (Fig. 7K). This group represents the majority of the EECs that are activated by linoleic acid (Fig. 7O). In the second EEC subgroup, linoleic acid increases cytoplasmic Ca^{2+} levels (Fig. 7L). However, the mitochondrial Ca^{2+} levels do not increase accordingly (Fig. 7L). As a result, the mitochondrial to cytoplasmic Ca^{2+} ratio is slightly lower post-linoleic acid stimulation (Fig. 7L). In the third EEC subgroup, linoleic acid does not increase cytoplasmic Ca^{2+} nor mitochondrial Ca^{2+} levels and the mitochondrial to cytoplasmic Ca^{2+} ratio remains the same (Fig. 7M).

Finally, we investigated whether and how gut microbiota regulates the EECs' nutrient response. We generated GF and CV *Tg(neurod1:Gcamp6f); Tg(neurod1:mitoRGECO)* zebrafish (Fig. 8A). We then stimulated the GF and CV zebrafish with linoleic acid and recorded how the GF and CV EECs responded to the linoleic stimulation (Fig. 8B-I). Our results indicate that, compared with CV zebrafish, the percentage of EECs that can be

activated by linoleic acid in GF zebrafish decreased (Fig. 8J). Within the activated EECs, the cytoplasmic Ca^{2+} amplitude remains the same between GF and CV groups (Fig. 8K). However, within the activated EECs, the mitochondrial Ca^{2+} amplitude significantly increased in the CV EECs (Fig. 8L). The same result is shown when we trace the temporal EEC cytoplasmic and mitochondrial Ca^{2+} levels in GF and CV zebrafish (Fig. 8M-R). In most CV EECs, nutrient stimulation activates both cytoplasmic and mitochondrial Ca^{2+} and increases the mitochondrial to cytoplasmic Ca^{2+} ratio (Fig. 8P-R). However, the nutrient induced EEC mitochondrial activation is significantly reduced in GF EECs (Fig. 8M-O). We did not detect nutrient induced mitochondrial-to-cytoplasmic Ca^{2+} ratio increase in GF EECs (Fig. 8M-O). Together, these results indicate that gut microbiota is critical in promoting EEC mitochondrial function and nutrient-induced EEC mitochondrial activation.

Discussion

In this study, by using transcriptomics, genetics, in vivo imaging, and gnotobiotic manipulation, we revealed that commensal microbiota colonization is critical in shaping EEC maturation and function during development(Fig. 9). Importantly, our data revealed that commensal microbiota colonization is essential in promoting mitochondrial activity and nutrient induced mitochondrial activation (Fig. 9). Selectively manipulating gut microbial signals to alter EEC mitochondrial function will provide new opportunities to change EEC vesicle secretion and EEC-neuronal communication.

The change of EEC mitochondrial activity during development.

Using in vivo imaging to track EECs during development in live zebrafish, our results revealed that EEC mitochondrial activity is dynamically regulated during development. It demonstrated that, shortly after commensal microbiota colonization, EECs increase both cellular and mitochondrial calcium activity. A phenomenon we referred to as the “EEC awakening”. After the EEC awakening, the EECs down-regulate their cytoplasmic calcium levels but up-regulate their mitochondrial-to-cytoplasmic Ca^{2+} ratio. As sensory cells, it is critical for EECs to maintain low cytoplasmic Ca^{2+} levels to enable a depolarization potential. When EECs sense nutrient stimulants, the calcium ion channel on the cell membrane or ER membrane will open [2]. Ca^{2+} from the extracellular space or the ER lumen will flux into the cytoplasm matrix following the Ca^{2+} gradient. The low cytoplasmic Ca^{2+} levels are, therefore, essential to generating the gradient to produce the Ca^{2+} peak to trigger the downstream cellular signaling events [36]. Maintaining the membrane potential or the low cytoplasmic Ca^{2+} levels consumes ATP [36]. ATP can be generated via glycolysis or through the tricarboxylic acid cycle (TCA cycle) and oxidative phosphorylation mediated by mitochondria [37]. It is well appreciated that mitochondrial and metabolic remodeling is a central feature of differentiation and reprograming events [17]. The mitochondrial oxidative metabolism is often suppressed in stem cells [38, 39]. The stem cells, including intestinal stem cells, rely on glycolysis to generate ATP [40, 41]. The mitochondria in stem cells remain functional [40]. However, stem cells possess multiple mechanisms to suppress mitochondrial activity [41-43]. Upon differentiation, mitochondrial activity increases [40, 41]. On one hand, the increased mitochondrial activity fuels the high metabolic demand of the differentiated cells. On the other hand, the

increased mitochondrial activity generates necessary signaling molecules such as reactive oxygen species (ROS) and biosynthetic metabolites through the TCA cycle to promote the differentiation process [44, 45]. The mitochondria-derived signaling molecules also promote epigenetic remodeling and modulate gene expression [46]. Using an *in vivo* EEC mitochondria imaging approach to trace the same zebrafish across different developmental time points, our results revealed that the immature EECs at 3 days post fertilization display low mitochondrial activity. When zebrafish develop and EECs start to be functional, mitochondrial activity increases. The increased mitochondrial activity may not only provide energy to fuel the EEC cellular process but also provide the signaling that is necessary for the EECs to mature and function. Interestingly, in addition to the change in mitochondrial activity, we also observed changes in intracellular mitochondrial distribution during development. Specifically, our results revealed the increased mitochondrial contents at the basal lateral membrane during the EEC maturation process. The increased mitochondrial distribution in the mature EECs is likely to match the ATP demand of the vesicle secretion process in the basal lateral membrane.

In addition to providing energy, mitochondria also function as an important calcium buffer. In response to extracellular stimulation, cytoplasmic Ca^{2+} levels increase. This increase in cytoplasmic Ca^{2+} is quickly dissipated into intracellular organelles, such as the ER or mitochondria. In most cells, mitochondrial calcium uptake is mediated by the mitochondrial calcium uniporter (MCU), a calcium transporter protein in the mitochondrial inner membrane. The electrochemical potential across the mitochondrial inner membrane, generated by the respiration chain reaction, is the major driving force that enables calcium influx into the mitochondrial matrix via MCU. Cytoplasmic and mitochondrial Ca^{2+} coupling have not been studied in EECs. Our studies revealed that, in response to nutrient stimulation, a subset of EECs increase cytoplasmic Ca^{2+} activity. Following the increase of cytoplasmic Ca2+ levels, the mitochondrial Ca^{2+} levels increase in most activated EECs. Though the cytoplasmic Ca^{2+} quickly returned to the basal level, most EEC mitochondrial Ca^{2+} was continuously maintained at a high level. Basal mitochondrial respiratory function might be the key to mediating Ca^{2+} flux into the mitochondrial matrix. The increase in mitochondrial Ca^{2+} will increase mitochondrial respiration to sustain the ATP production that is required for the EEC vesicle secretion in response to the nutrient stimulants [20]. Consistent with these findings, the concentrated mitochondrial contents in the basal membrane, where vesicle secretion occurs, support the hypothesis that mitochondrial activation assists with vesicle secretion in mature EECs.

The change of EEC morphology during development

In addition to the change in EEC mitochondrial activity, another hallmark of EEC maturation, revealed by our study, is the change in EEC morphology. Our study illustrated for the first time that immature EECs possess dynamic and active actin filaments in the basal membrane. However, the actin filaments disappeared in mature EECs. Instead, some mature EECs formed an elongated basal lateral membrane process, a structure that resembles the “neuropod” reported in previous mammalian studies [3]. Previous studies demonstrated that the neuropod structure enriches the neurofilaments and mitochondria [47]. EECs use neuropods to form synaptic connections with the underlying nerve terminates, including the vagal sensory nerve [3, 4]. What regulates the EEC

neuropod formation and guides the EEC-neuronal synaptic connection remains unknown. In developing neurons, neurites form actin-supported extensions known as growth cones which seek synaptic targets [48]. Formation of the pre- and post-synaptic structures disables the filipodium-enriched actin structure at the leading age [49]. Can the EECs form a growth cone-like structure to find their targets and form synaptic connections with the neurons? Our results revealed that the immature EECs form thin actin-based elements in the basal lateral membrane. These thin actin elements are similar to the filopodia projections found in the developing neuron axon growth cone. In some EECs, these thin actin filaments are replaced by the neuropod-like structure when EECs mature. This morphology evidence supported the hypothesis that the immature EECs may use active actin filaments to find the synaptic targets and form the synaptic connections with the underlying neurons. Establishing the EEC-neuronal connection will facilitate the EECs to transmit the ingested nutrients to the nervous system.

How does gut microbiota regulate EEC maturation and mitochondrial function?

A major finding revealed by our study is that commensal microbiota colonization is critical in supporting EEC maturation and promoting EEC mitochondrial function. Our results established that microbiota colonization during early development might be critical in establishing the organisms' appropriate nutrient-sensing function via promoting EEC maturation. Our results also suggested that postnatal microbiota colonization might be critical in promoting the formation of EEC-vagal neuronal communication as the commensal microbiota colonization promotes the formation of the neuropod-like structure. The EEC-vagal neuronal connection is essential in mediating gut-brain nutrient sensing. Gut microbiota can therefore modulate EEC or EEC-vagal communication to regulate brain nutrient perception and feeding behavior. Our results revealed that the EECs remain in an immature state and exhibit low mitochondrial activity when commensal microbiota is absent. Disrupting the commensal microbiota colonization or inhibiting the formation of the healthy postnatal microbiome may produce devasting effects on gut nutrient perception and metabolic regulation. The formation of the postnatal gut microbial community is influenced by many factors (maternal microbiome, delivery method, milk-feeding vs formula feeding). Previous research showed that disrupting the infant microbiome through antibiotic exposure results in many side effects, including obesity and weight gain later in life [50]. The EECs are critical in sensing ingested nutrients and maintaining homeostasis [2]. Our study suggests that disrupting the commensal microbial community early in life will change the EECs' function and maturity, which may bring long-lasting effects in changing how the body responds to ingested nutrients. This may also affect energy homeostatic control later in life.

The intestine epithelium undergoes a self-renew process throughout life. The new EECs continuously rise from intestinal stem cells, which differentiate and mature into functional EECs [51]. The changes in EEC morphology and mitochondrial function observed in our study may also be involved in the EEC maturation process in adult homeostatic conditions. It is possible that gut microbiota also governs the EECs' differentiation and maturation process in this scenario. Even though the microbial community in the adult intestine is different from the infant gut microbiota [9], varying microbial signals may target the same signaling pathway to drive the EEC maturation process.

Methods

Zebrafish strains and husbandry

All zebrafish experiments conformed to the US Public Health Service Policy on Humane Care and Use of Laboratory Animals, using protocol number 2021A00000091 approved by the Institutional Animal Care and Use Committee of the Ohio State University. Conventionally-reared adult zebrafish were reared and maintained on a recirculating aquaculture system using established methods [28]. For experiments involving conventionally-raised zebrafish larvae, adults were bred naturally in system water and fertilized eggs were transferred to 100mm petri dishes containing ~25mL of egg water at approximately 6 hours post-fertilization. The resulting larvae were raised under a 14h light/10h dark cycle in an air incubator at 28°C at a density of 2 larvae/ml water. All the experiments performed in this study ended at 7dpf unless specifically indicated. The zebrafish lines used in this study are listed in Table 1. All lines were maintained on a EKW background.

Gnotobiotic zebrafish husbandry

For experiments involving gnotobiotic zebrafish, we used our established methods to generate germ-free zebrafish using natural breeding (ref.) with the following exception: Gnotobiotic Zebrafish Medium (GZM) with antibiotics (AB-GZM) was supplemented with 50 µg/ml gentamycin (Sigma, G1264). Germ-free zebrafish eggs were maintained in cell culture flasks with GZM at a density of 1 larvae/ml. From 3 dpf to 7 dpf, 60% daily media change, and newborn fish food (Ultra Fresh Ltd.) feeding were performed as described [28].

To generate conventionalized zebrafish, 15 mL filtered system water (5µm filter, SLSV025LS, Millipore, final concentration of system water ~30%) was inoculated to flasks containing germ-free zebrafish in GZM at 3 dpf when the zebrafish normally hatch from their protective chorions. The same feeding and media change protocol was followed as for germ free zebrafish. Microbial colonization density was determined via Colony Forming Unit (CFU) analysis. To analyze the effect of high fat feeding on intestinal bacteria colonization, dissected digestive tracts were dissected and pooled (5 guts/pool) into 1mL sterile phosphate buffered saline (PBS) which was then mechanically disassociated using a Tissue-Tearor (BioSpec Products, 985370). 100 µL of serially diluted solution was then spotted on a Tryptic soy agar (TSA) plate and cultured overnight at 30°C under aerobic conditions.

Zebrafish EEC RNA sequencing analysis

The zebrafish EEC RNA sequencing data was generated in our previous study (ref.). This dataset can be accessed at GSE151711. Conventionalized (CV) and germ-free (GF) *TgBAC(cldn15la:EGFP); Tg(neurod1:TagRFP)* ZM000 fed zebrafish larvae were derived and reared using the published protocol [28] for Flow Activated Cell Sorting (FACS) to isolate zebrafish EECs and other IECs. The protocol for FACS was adopted from a previous publication [27]. Replicate pools of 50-100 double transgenic

TgBAC(cldn15la:EGFP); Tg(neurod1:TagRPF) zebrafish larvae were euthanized with Tricaine and washed with deyolking buffer (55 mM NaCl, 1.8 mM KCl and 1.25 mM NaHCO3) before they were transferred to dissociation buffer [HBSS supplemented with 5% heat-inactivated fetal bovine serum (HI-FBS, Sigma, F2442) and 10 mM HEPES (Gibco, 15630–080)]. Larvae were dissociated using a combination of enzymatic disruption using Liberase (Roche, 05 401 119 001, 5 µg/mL final), DNaseI (Sigma, D4513, 2 µg/mL final), Hyaluronidase (Sigma, H3506, 6 U/mL final) and Collagenase XI (Sigma, C7657, 12.5 U/mL final) and mechanical disruption using a gentleMACS dissociator (Miltenyi Biotec, 130-093-235). 400 µL of ice-cold 120 mM EDTA (in 1x PBS) wwas added to each sample at the end of the dissociation process to stop the enzymatic digestion. Following addition of 10 mL Buffer 2 [HBSS supplemented with 5% HI-FBS, 10 mM HEPES and 2 mM EDTA], samples were filtered through 30 µm cell strainers (Miltenyi Biotec, 130-098-458). Samples were then centrifuged at 1800 rcf for 15 minutes at room temperature. The supernatant was decanted, and cell pellets were resuspended in 500 µL Buffer 2. FACS was performed with a MoFlo XDP cell sorter (Beckman Coulter) at the Duke Cancer Institute Flow Cytometry Shared Resource. Single-color control samples were used for compensation and gating. Viable EECs or IECs were identified as 7-AAD negative.

Samples from three independent experimental replicates were performed. 250-580 EECs (n=3 for each CV and GF group) and 100 IECs (n=3 for each CV and GF group) from each experiment were used for library generation and RNA sequencing. Total RNA was extracted from cell pellets using the Argencourt RNAdvance Cell V2 kit (Beckman) following the manufacturer's instructions. RNA amplification prior to library preparation had to be performed. The Clontech SMART-Seq v4 Ultra Low Input RNA Kit (Takara) was used to generate full-length cDNA. mRNA transcripts were converted into cDNA through Clontech's oligo(dT)-priming method. Full length cDNA was then converted into an Illumina sequencing library using the Kapa Hyper Prep kit (Roche). In brief, cDNA was sheared using a Covaris instrument to produce fragments of about 300 bp in length. Illumina sequencing adapters were then ligated to both ends of the 300bp fragments prior to final library amplification. Each library was uniquely indexed allowing for multiple samples to be pooled and sequenced on two lanes of an Illumina HiSeq 4000 flow cell. Each HiSeq 4000 lane could generate >330M 50bp single end reads per lane. This pooling strategy generated enough sequencing depth (~55M reads per sample) for estimating differential expression. Sample preparation and sequencing was performed at the GCB Sequencing and Genomic Technologies Shared Resource.

Zebrafish RNA-seq reads were mapped to the danRer10 genome using HISAT2(Galaxy Version 2.0.5.1) using default settings. Normalized counts and pairwise differentiation analysis were carried out via DESeq2. The significance threshold of $p < 0.05$ was used for comparison.

Immunofluorescence staining

Whole mount immunofluorescence staining was performed as previously described [24]. In brief, ice cold 2.5% formalin was used to fix zebrafish larvae overnight at 4°C. The samples were then washed with PT solution (PBS+0.75%Triton-100). The skin and

remaining yolk were then removed using forceps under a dissecting microscope. The deyolked samples were then permeabilized with methanol for more than 2 hrs at -20°C. Samples were then blocked with 4% BSA at room temperature for more than 1 hr. The primary antibody was diluted in PT solution and incubated at 4°C for more than 24 hrs. Following primary antibody incubation, the samples were washed with PT solution and incubated overnight with secondary antibody with Hoechst 33342 for DNA staining. Imaging was performed with Nikon AXR confocal using the 20× of 40× water immersion lens. The primary antibodies were listed in Table 1. The secondary antibodies in this study were from Alexa Fluor Invitrogen were used at a dilution of 1:250.

Live imaging and image analysis

The zebrafish larvae were anesthetized with Tricaine methanesulfonate (MS222) and were mounted in the 35mm confocal dish using 1% low-melting-Agar. All the in vivo imaging were performed using the Nikon AXR confocal. When imaging the EEC cellular and mitochondrial calcium activity using the *Tg(neurod1:Gcamp6f); Tg(neurod1:mitoRGECO)* zebrafish, the zebrafish were not anesthetized due to the effects of Tricaine in activating EECs. In the developmental tracing experiments, after imaging, the zebrafish were dug out of the Agar, placed in 6-well plate, and returned to the incubator until the next imaging time point. In the experiments when the temporal EEC activity was traced, the images were collected using the resonate scanner. It takes less than 10 seconds to collect the whole intestinal z-stack. The interval of time frames is 10 seconds. In the experiments when the nutrient stimulants were applied. A small window was cut in front of the zebrafish which allows the zebrafish mouse to be exposure. First, the zebrafish intestine was imaged before the stimulants were applied to assess the basal line EEC activity. After collecting the basal EEC time lapse images. The image collection was pulsed to allow adding the nutrient stimulation. The egg water in the confocal dish was removed. 1ml nutrient stimulate solution was delivered into the window in front of the zebrafish. After the nutrient stimulation was applied, the image acquisition process resumes. The time lapse images were collected to assess the nutrient induced EEC activation. For the image analysis to assess the EEC calcium activity, the images were first aligned using the Nikon NLS element software. The threshold was defined using the Gcamp channel to perform segmentation of the individual EECs and identify individual EEC units. The non-EECs were filtered out via the shape criteria, the fluorescence intensity, and the size. The individual EEC units in different time frames were traced and tracked via the NLS element 3D-object tracing software. The mean fluorescence intensity of the individual EEC in each time frame will be calculated. The cluster 3.0 software was used to perform the clustering analysis of the EECs that exhibit different temporal calcium dynamics.

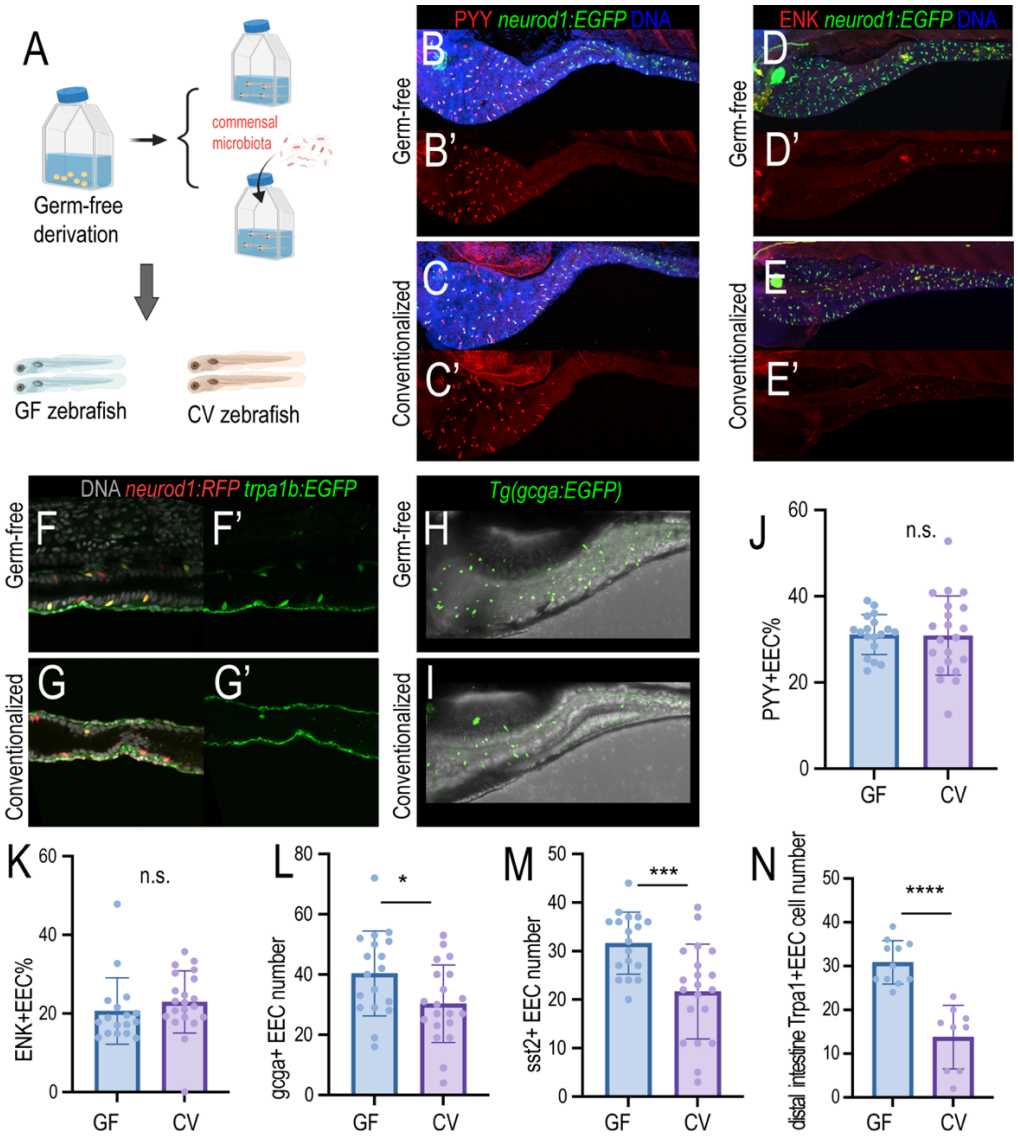
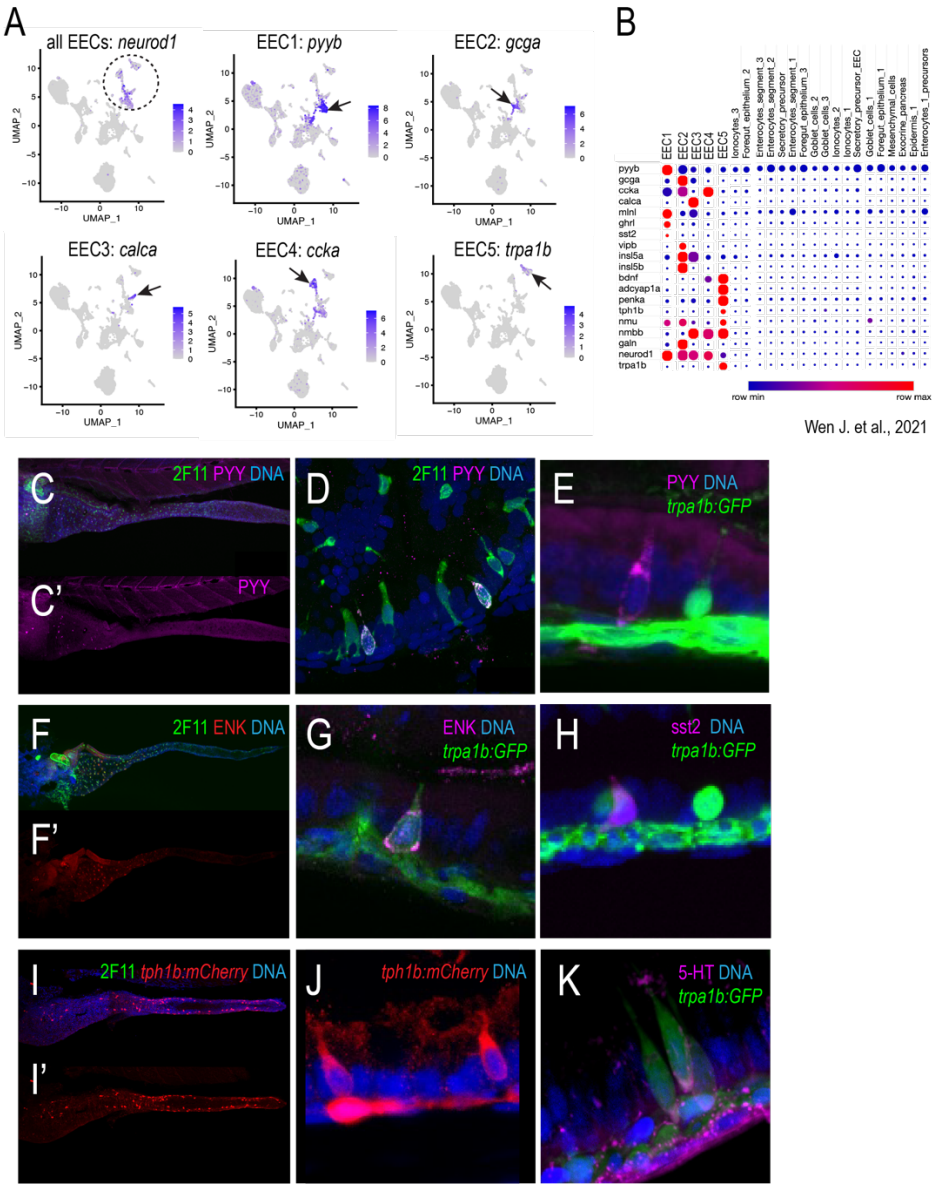
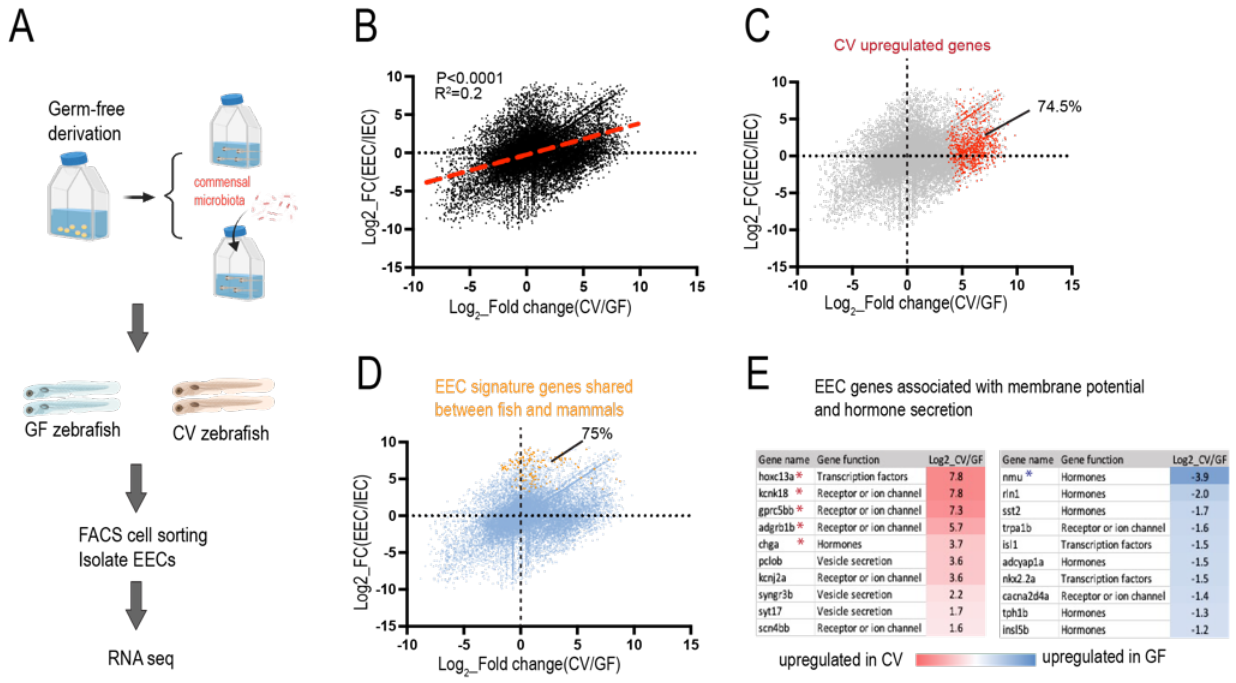


Figure 1. Gut microbiota modulates the EEC subtype. (A) Gnotobiotic zebrafish experimental procedure to examine the effects of gut microbiota on EEC subtype formation. Commensal microbiota was colonized at 3pdf and the zebrafish were fixed at 7dpf for immunofluorescence staining. (B-C') Confocal projection of the representative germ-free (GF) and conventionalized (CV) zebrafish intestine. The total EECs were labeled by the *Tg(neurod1:EGFP)* transgene (green), and the PYY+EECs were labeled via the PYY antibody (ref). (D-E') Confocal projection of the representative germ-free (GF) and conventionalized (CV) zebrafish intestine showing the ENK+ EECs. (F-G') Confocal projection of the representative germ-free (GF) and conventionalized (CV) zebrafish intestine showing the Trpa1+ EECs in the distal intestine. (H-I) Confocal projection of the representative germ-free (GF) and conventionalized (CV) zebrafish intestine showing the gcga+ EECs. (J-N) Quantification of the PYY+EECs, ENK+EECs, gcga+EECs, sst2+EECs and the Trpa1+EECs in GF and CV zebrafish. Student t-tests were used for statistical analysis. Each dot represents individual zebrafish. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.



Supplemental Figure 1. The EEC subtypes in zebrafish larvae. (A) UMAP plots of the zebrafish intestine single-cell RNA sequencing showing the zebrafish EECs and the five EEC subtypes in zebrafish larvae. The zebrafish scRNA dataset is from Wen J. et al., 2021. (B) The hormone profiles in the five zebrafish EEC subtypes. (C-E) Immunofluorescence staining of the PYY+EEC subtype. Note that the PYY+EECs are distributed in the proximal zebrafish intestine (C-C'). It overlaps with the secretory cell marker 2F11 (D) but does not overlap with the marker for other EEC subtypes, such as trpa1b (E). (F-K) Immunofluorescence staining of the Trpa1+EEC subtype. The single-cell RNA seq data above demonstrate that the Trpa1+EECs (EEC5) express the peptide enkephalin (ENK) and the enzyme that synthesizes serotonin (tph1b). (F-G) Immunofluorescence staining of ENK confirms that only Trpa1+EECs express ENK (G). Interestingly, ENK is only expressed in the Trpa1+EECs in the proximal intestine (F-F'). (H) Trpa1+EECs do not express sst2, a marker for the EEC subtype 1. (I-J) Tph1b is

expressed in the EECs. (K) Immunofluorescence staining showing part of the Trpa1+EECs express 5-HT.



Gene name	Gene function	Log2_CV/GF
hoxc13a *	Transcription factors	7.8
kcnk18 *	Receptor or ion channel	7.8
gprc5bb *	Receptor or ion channel	7.3
adgrb1b *	Receptor or ion channel	5.7
chga *	Hormones	3.7
pclob	Vesicle secretion	3.6
kcnj2a	Receptor or ion channel	3.6
syngr3b	Vesicle secretion	2.2
syt17	Vesicle secretion	1.7
scn4bb	Receptor or ion channel	1.6

Gene name	Gene function	Log2_CV/GF
nmu *	Hormones	-3.9
rln1	Hormones	-2.0
sst2	Hormones	-1.7
trpa1b	Receptor or ion channel	-1.6
isl1	Transcription factors	-1.5
adcyap1a	Hormones	-1.5
nkx2.2a	Transcription factors	-1.5
cacna2d4a	Receptor or ion channel	-1.4
tph1b	Hormones	-1.3
insl5b	Hormones	-1.2

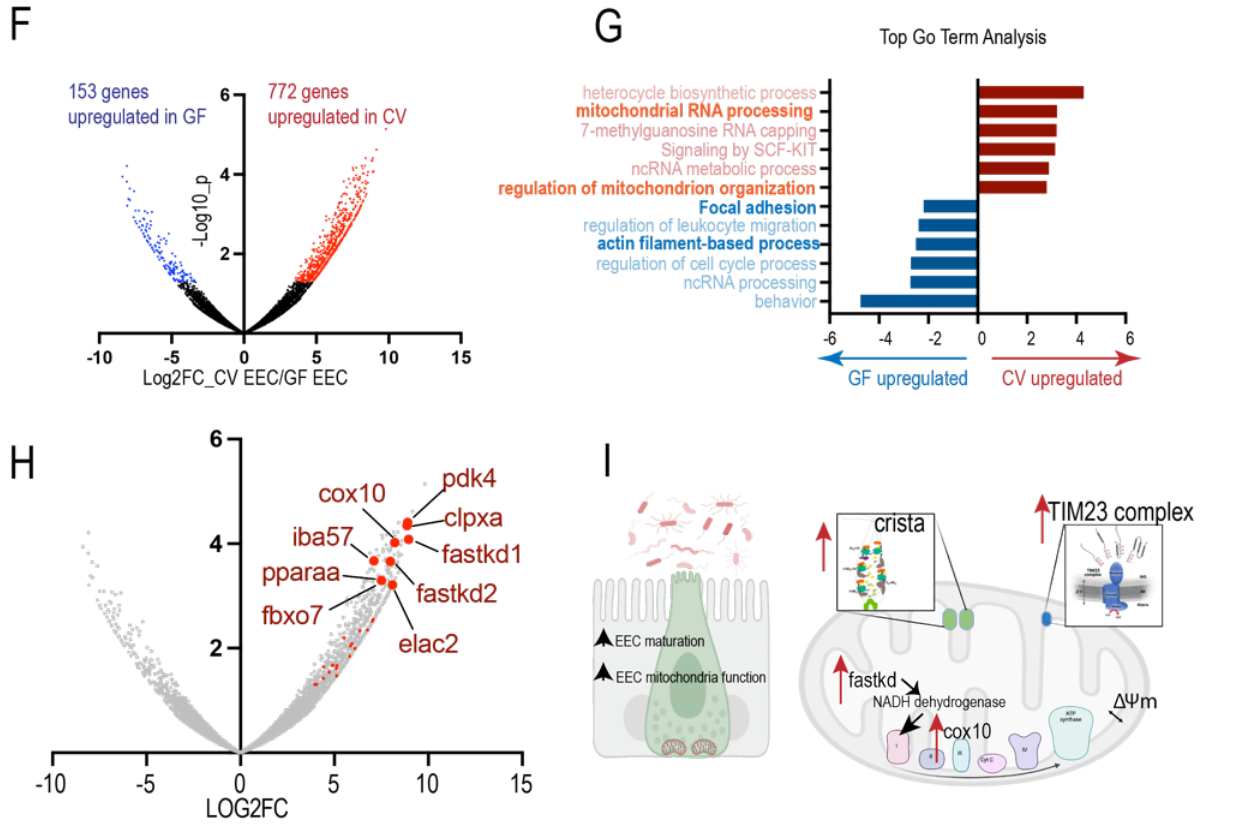


Figure 2. Gut microbiota promotes EEC maturation and mitochondrial function. (A) Transcriptomic analysis of the FACS sorted EECs from GF and CV zebrafish. (B) Positive correlation between the genes that are upregulated in CV (X-axis) and the genes that are enriched in EECs (Y-axis). (C) Among the genes that are significantly upregulated in CV (red color), 74.5% are enriched in the EECs. (D) 75% of the EECs signature genes shared between zebrafish and mammals are upregulated in CV. (E) The differential expression of the EEC signature genes that encode hormone peptides or are involved in membrane potential in GF and CV conditions. * Indicates that the genes are significantly upregulated in the GF or CV conditions. (F) The volcano plot shows the genes that are significantly upregulated in CV or GF. (G) Go-term analysis of the CV or GF upregulated genes. (H) The volcano plot shows the genes that are involved in mitochondrial function. Many of the genes that are associated with mitochondrial regulation are among the most significantly upregulated genes in CV EECs. (I) The model figure shows that commensal microbiota colonization promotes EEC maturation and EEC mitochondrial function.

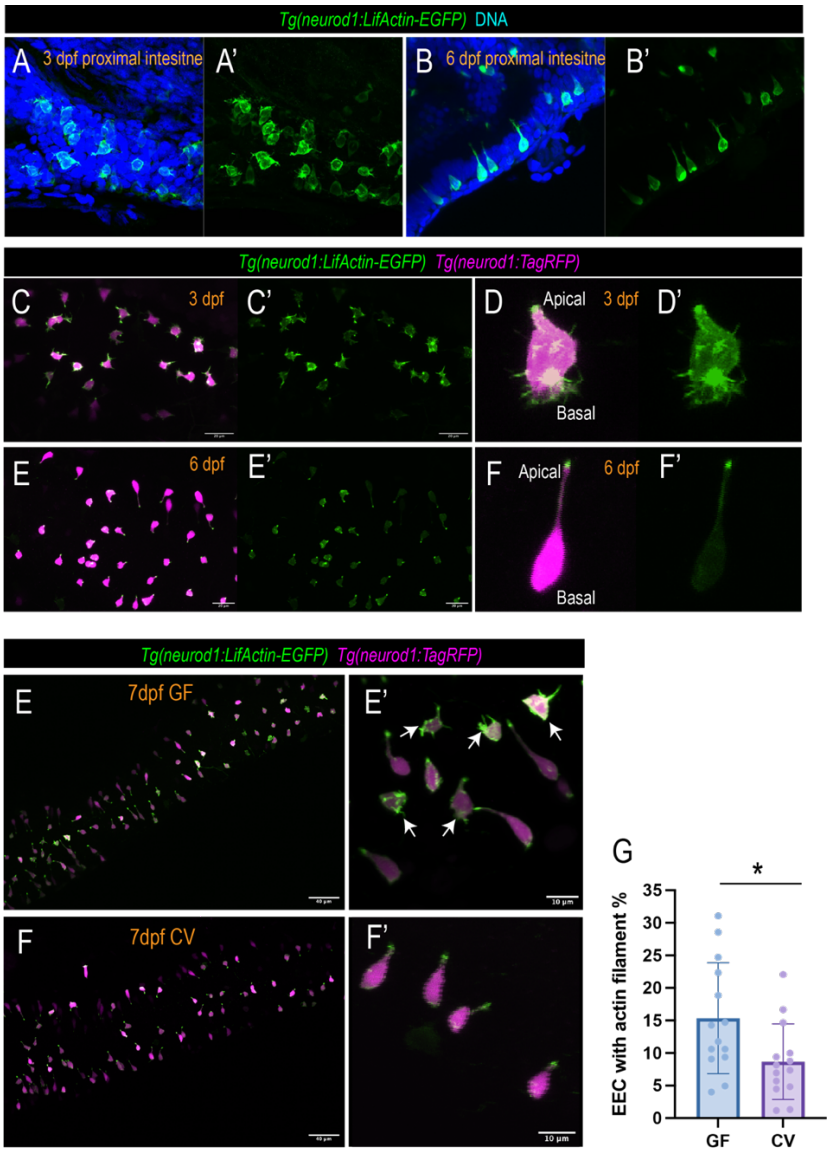
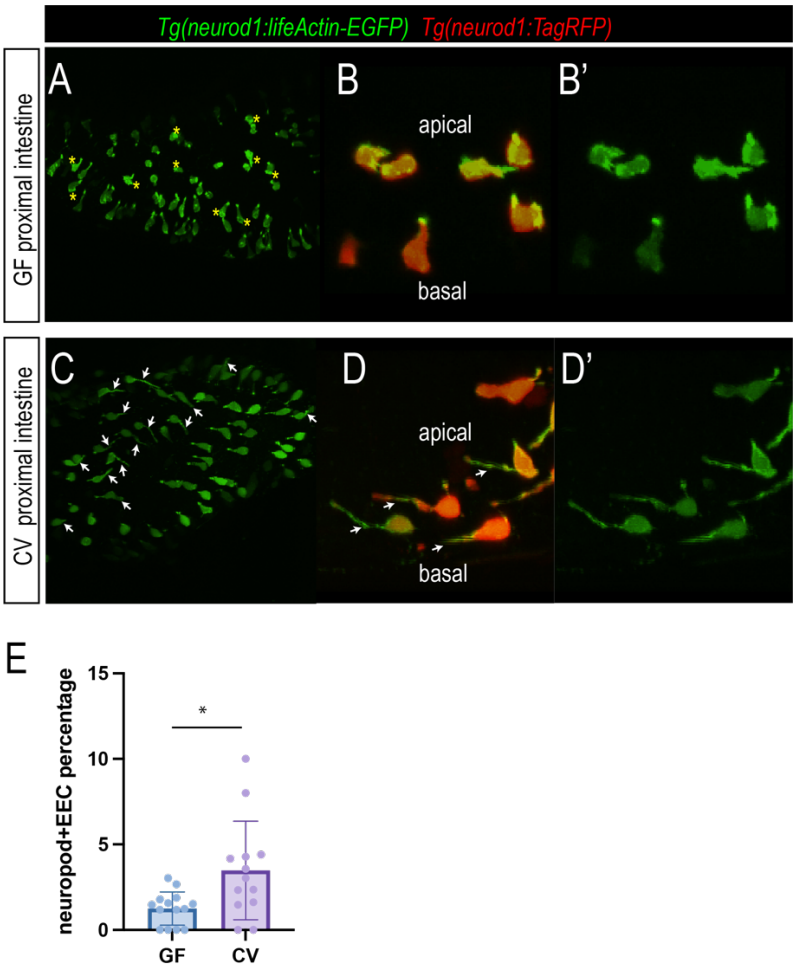


Figure 3. EECs change morphology during development in a microbial-dependent manner. (A-B') Confocal projections of the proximal intestine *Tg(neurod1:lifeActin-EGFP)* zebrafish at 3dpf and 6dpf in the fixed samples. The EECs in the 3 dpf but not 6 dpf intestine exhibit thin actin filaments in the basal lateral membrane. (C-E') Live imaging traces the same zebrafish's EECs at 3dpf and 6dpf. The EEC actin filaments are labeled via the *Tg(neurod1:lifeActin-EGFP)*. (D-F') Zoom out view showing the typical 3dpf EEC and 6dpf EEC. Note that at 3dpf, active actin filaments are observed at the basal lateral membrane. At 6dpf, the actin filaments are only enriched in the apical brush border. (E-F') Confocal projection of the 7 dpf GF and CV zebrafish EECs. The EEC actin filaments are labeled via the *Tg(neurod1:lifeActin-EGFP)*. Note that the active basal lateral actin filaments remained in a subpopulation of EECs in GF zebrafish. (G) Quantification of the percentage of EECs with actin filaments in GF and CV conditions. Each dot represents an individual zebrafish. Student-T test was used in G. *P<0.05.



Supplemental Figure 2. Commensal microbiota colonization promotes the formation of "neuropod"-like structure in EECs. (A-D') Confocal projections of the GF and CV *Tg(neurod1:lifeActin-EGFP)* zebrafish. The yellow stars in A indicate EECs with thin actin filaments at the basal lateral membrane. The White arrows in C and D indicate the "neuropod" like elongated basal lateral membrane protrusions in CV EECs. (E) Quantification of the EEC percentage that has "neuropod" like structure in GF and CV conditions. Student T-test was used in E. Each dot represents an individual zebrafish. * $P<0.05$.

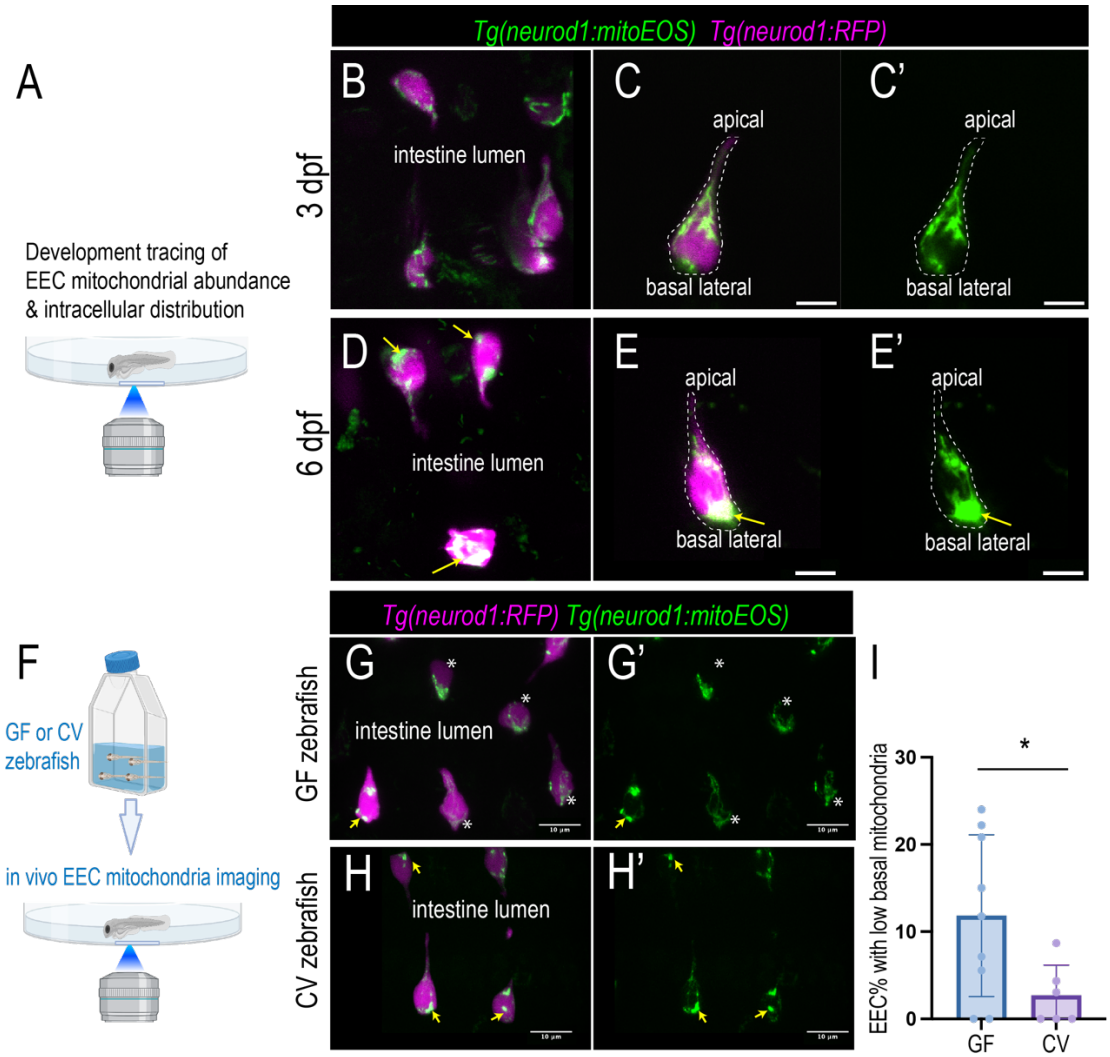
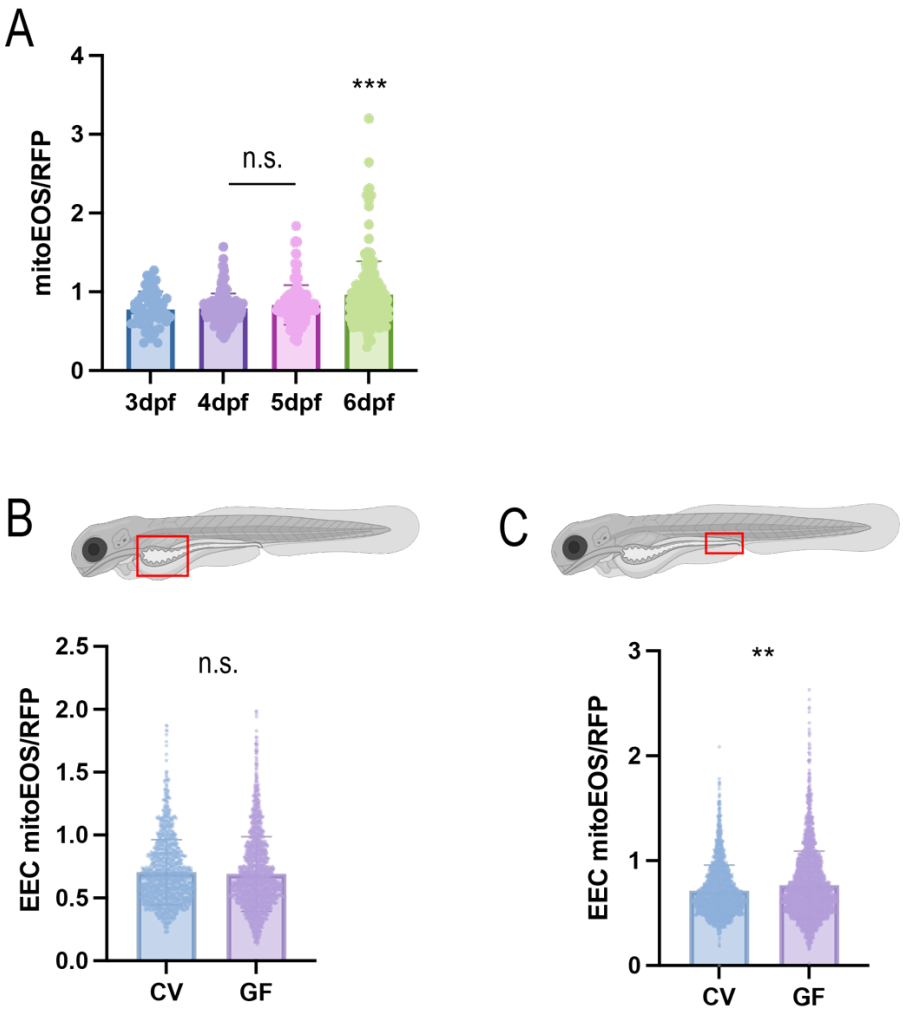


Figure 4. Commensal microbiota colonization promotes mitochondria accumulation at the EEC basal lateral membrane. (A) In vivo imaging to trace the EEC mitochondrial abundance and the intracellular mitochondria distribution in the same zebrafish. (B-E') Confocal projections of the typical EECs at 3dpf and 6dpf. The mitochondria are labeled via the *Tg(neurod1:mitoEOS),* and the EECs are labeled via the *Tg(neurod1:RFP)*. Note that at 3dpf, the mitochondria are evenly distributed in the EEC cytoplasm, and the mitochondria contents at the basal lateral membrane are low. At 6dpf, the mitochondria distribution exhibits a hot spot pattern. In most EECs, a spot at the basal lateral membrane (yellow arrows in D-E') contains highly abundant mitochondria. (F) In vivo imaging to examine the mitochondria intracellular distribution in 7dpf GF and CV zebrafish. (G-H') Confocal projections of the typical EECs in GF and CV zebrafish. Note that many EECs in GF zebrafish have low mitochondria contents at the basal lateral membrane (white stars in G and G'). Most EECs in CV zebrafish exhibit hot spot basal lateral membrane mitochondrial distribution patterns (yellow arrows in H and H'). (I) Quantification of the percentage of EECs with low mitochondria contents at the basal lateral membrane in 7dpf GF and CV zebrafish. Each dot represents an individual zebrafish. Student T-test was used. * $P<0.05$.



Supplemental Figure 3. Gut microbiota did not alter the proximal intestine mitochondria abundance. (A) Quantification of the intracellular mitochondria abundance in the 3dpf to 6dpf zebrafish EECs. The mitochondrial abundance is represented by the *neurod1:mitoEOS* and *neurod1:RFP* fluorescence ratio in individual EECs. Each dot represents an EEC. 4 zebrafish were analyzed. (B-C) Quantification of the intracellular mitochondria abundance in the proximal and distal intestines of the GF and CV zebrafish. The mitochondrial abundance is represented by the *neurod1:mitoEOS* and *neurod1:RFP* fluorescence ratio in individual EECs. Each dot represents an EEC. More than 7 zebrafish in GF and CV groups were quantified. One-Way Anova followed by Tukey post-test was used in A. Student T-test was used in B and C. *** $P<0.001$, ** $P<0.01$.

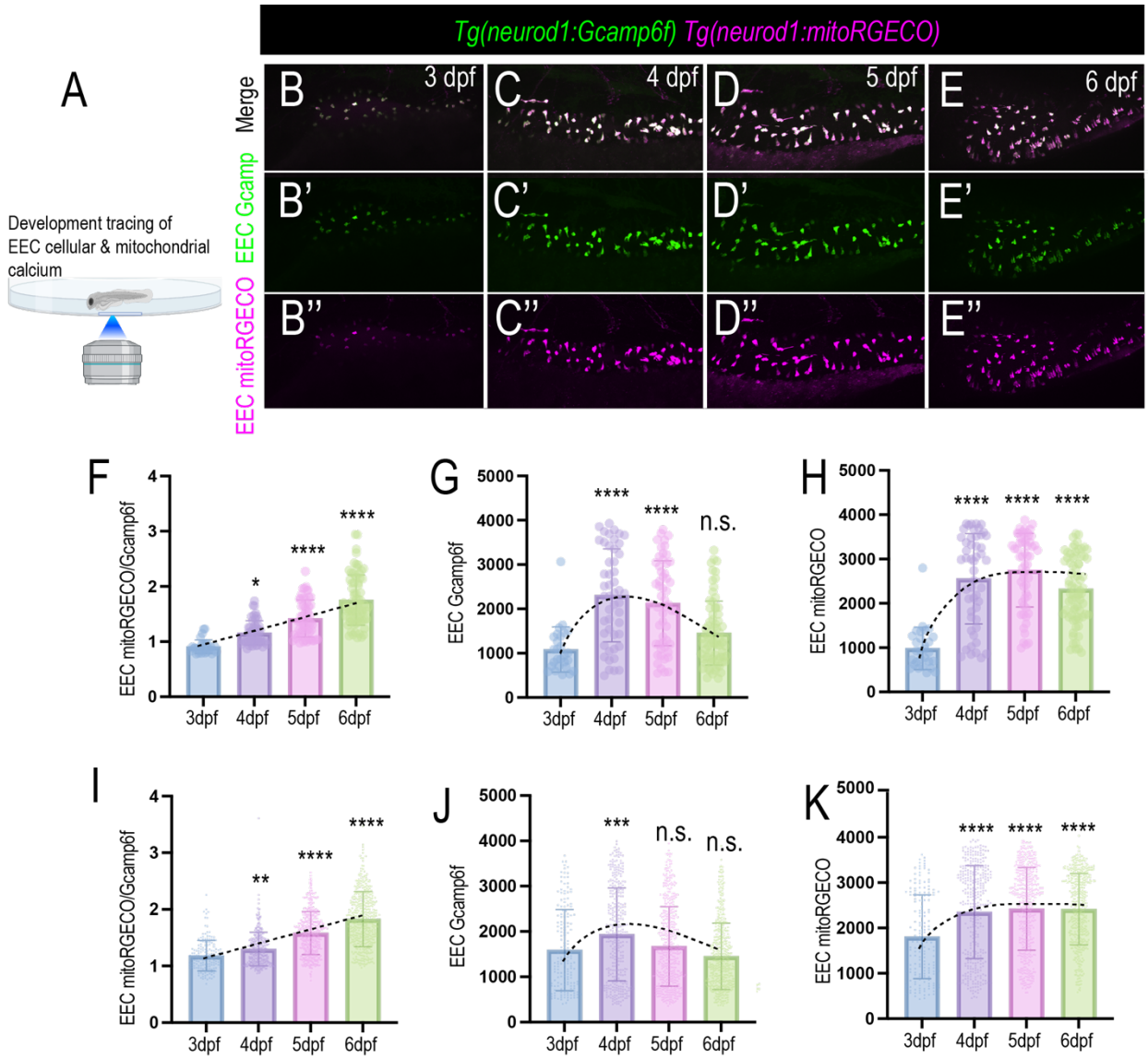


Figure 5. The change of EEC mitochondrial activity during development. (A) In vivo imaging to trace the EEC cellular and mitochondria activity in the same zebrafish from 3dpf to 6dpf. (B-E'') Confocal projections of the same *Tg(neurod1:Gcamp6f); Tg(neurod1:mitoRGECO)* zebrafish at 3dpf, 4dpf, 5dpf, and 6dpf. The EEC cytoplasmic Ca^{2+} level is represented via the Gcamp6f fluorescence (green). The EEC mitochondrial Ca^{2+} level is displayed through mitoRGECO fluorescence (red). (F-H) Quantification of the EEC mitochondria-to-cytoplasmic Ca^{2+} ratio, cytoplasmic Ca^{2+}, and mitochondrial Ca^{2+} in the zebrafish represented in B-E''. (I-K) Combined quantification of 10 zebrafish EECs mitochondria-to-cytoplasmic Ca^{2+} ratio, cytoplasmic Ca^{2+}, and mitochondrial Ca^{2+} . Each dot in F-K represents an individual EEC. One-Way Anova followed by Tukey's post test was used in F-K for statistical analysis. *p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

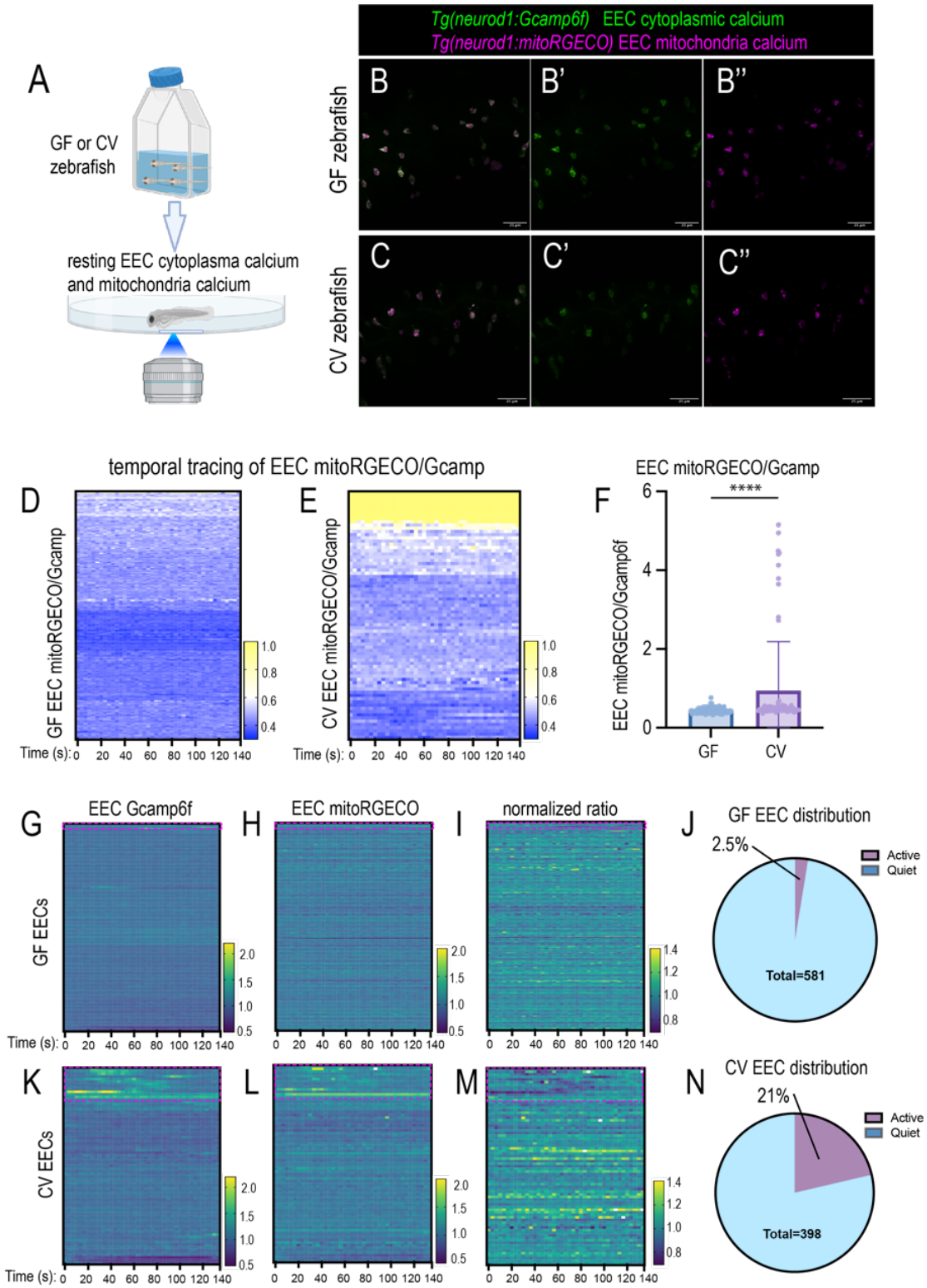
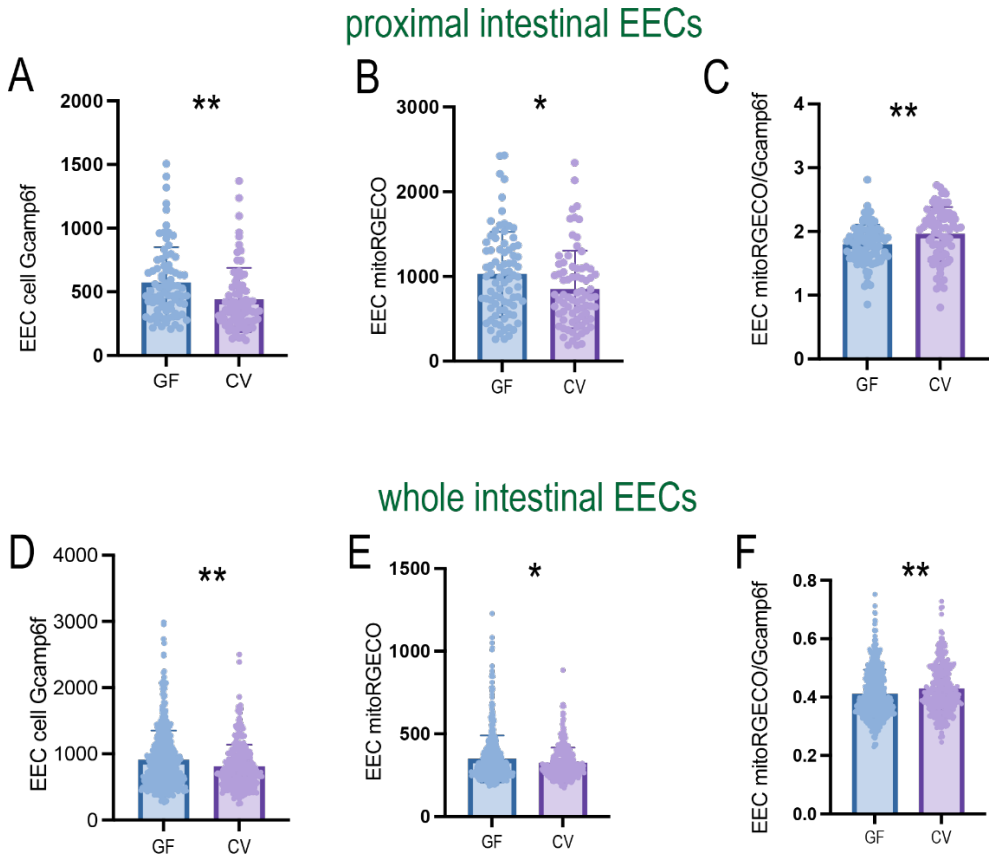


Figure 6. Commensal microbiota colonization alters the resting EEC cytoplasm and mitochondria calcium activity. (A) In vivo imaging to analyze the 7dpf GF and CV zebrafish EEC cellular and mitochondrial activity. (B-C'') Confocal projection of the GF and CV *Tg(neurod1:Gcamp6f); Tg(neurod1:mitoRGECO)* zebrafish. Note that the

absolute EEC Gcamp fluorescence is relatively higher in GF EECs. The EEC mitoRGECO/Gcamp ratio is lower in GF EECs. (D-E) Analyze the absolute EEC mitoRGECO/Gcamp ratio in GF and CV zebrafish on a temporal scale. Each line in D and E represents an individual EEC. (F) Quantification of the absolute EEC mitoRGECO/Gcamp ratio in GF and CV zebrafish at time 0. (G-M) Analyze the relative EEC Gcamp, EEC mitoRGECO, and EEC mitoRGECO/Gcamp ratio in GF and CV zebrafish on a temporal scale. The EEC Gcamp, EEC mitoRGECO, and EEC mitoRGECO/Gcamp ratio at each time point were normalized to t0. These quantification data sets reveal the dynamic change of EEC cellular and mitochondrial activity in GF and CV conditions. Each line in G-M represents an individual EEC. The red circles in G-M indicate the EECs that exhibit Gcamp fluorescence fluctuation. These EECs that display dynamic Gcamp fluorescence fluctuation were also referred to as active EECs in J and N. (J-N) Quantification of the percentage of the quiet and active EECs in GF and CV zebrafish. StudentT-test was used in F for statistical analysis. **** P<0.0001.



Supplemental Figure 4. Commensal microbiota colonization alters the resting EEC cytoplasm and mitochondria calcium activity. In this data set, GF or CV *Tg(neurod1:Gcamp6f);Tg(neurod1:mitoRGECO)* zebrafish were mounted on the confocal microscope, and a z-stack image at the intestinal region was collected for each zebrafish. No time-lapse tracing was performed. (A-C) Quantification of absolute Gcamp, mitoRGECO, and mitoRGECO/Gcamp ratio in GF and CV zebrafish proximal intestinal

EECs. (D-F) Quantification of absolute Gcamp, mitoRGECO, and mitoRGECO/Gcamp ratio in GF and CV zebrafish whole intestinal EECs. *p<0.05, ** p<0.01.

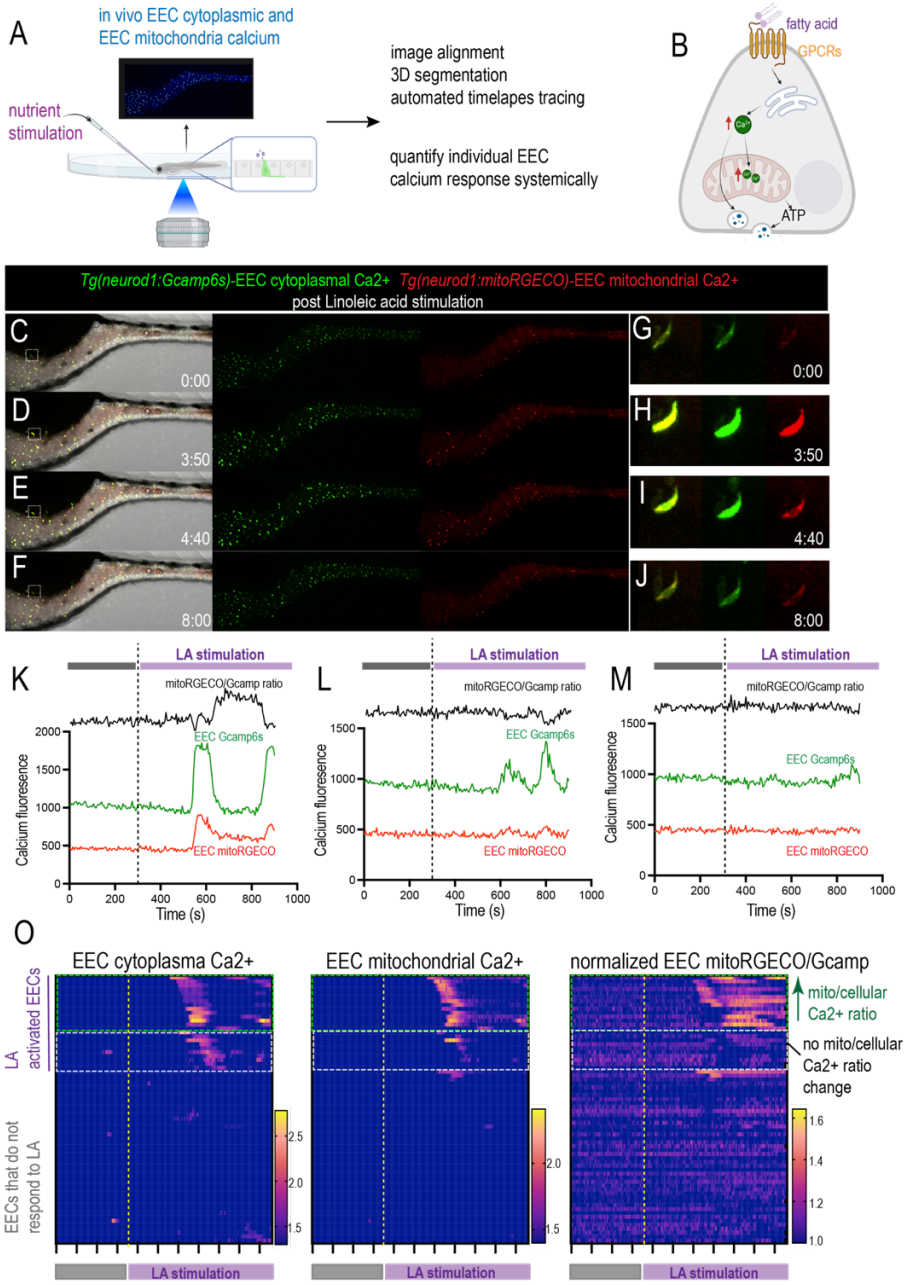


Figure 7. Analyze individual EEC cellular and mitochondrial activity in response to nutrient stimulation in live zebrafish. (A) Image and analyze the EEC cellular and mitochondrial calcium activity before and after nutrient stimulation using the confocal microscope. (B) The model figure shows how fatty acid increases both cytoplasmic and mitochondrial Ca^{2+}. Long-chain fatty acids, such as linoleic acid (LA), binds the fatty acid receptor at the cell membrane. Activating the fatty acid receptor induces Ca^{2+} release from the ER and increases the cytoplasmic Ca^{2+}. The increased cytoplasmic Ca^{2+} is then translocated into the mitochondrial matrix, which increases the mitochondrial matrix's

Ca^{2+} levels. The increased mitochondrial Ca^{2+} promotes ATP production and powers EEC vesicle secretion. (C-F) Time-lapse images of the EECs post linoleic acid stimulation. The EEC cytoplasmic Ca^{2+} was labeled by Gcamp6f (green), and the EEC mitochondrial Ca^{2+} was labeled by mitoRGECO (red). (G-J) Zoom-out view shows the Gcamp and mitoRGECO fluorescence change of an individual EEC in C-F (white box). (K-M) Analysis of fluorescence change of Gcamp, mitoRGECO, and mitoRGECO/Gcamp ratio in three different types of EECs before and after linoleic acid stimulation. (K) represents the EEC subtype which increases both cytoplasmic and mitochondrial Ca^{2+} in response to linoleic acid stimulation. (L) represents the EEC subtype which only increases cytoplasmic but not mitochondrial Ca^{2+} in response to linoleic acid stimulation. (M) represents the EEC subtype which does not increase cytoplasmic nor mitochondrial Ca^{2+} in response to linoleic acid stimulation. (O) Analysis of fluorescence change of Gcamp, mitoRGECO, and mitoRGECO/Gcamp ratio of 68 EECs in one zebrafish before and after linoleic acid stimulation. The EECs that increase cytoplasmic Ca^{2+} were defined as “LA activated EECs”. The EECs that increase both cytoplasmic and mitochondrial Ca^{2+} and exhibit increased mitochondria-to-cytoplasm Ca^{2+} ratio was circled in a yellow box. The EECs that increase only cytoplasmic Ca^{2+} but not mitochondrial Ca^{2+} were circled in a white box.

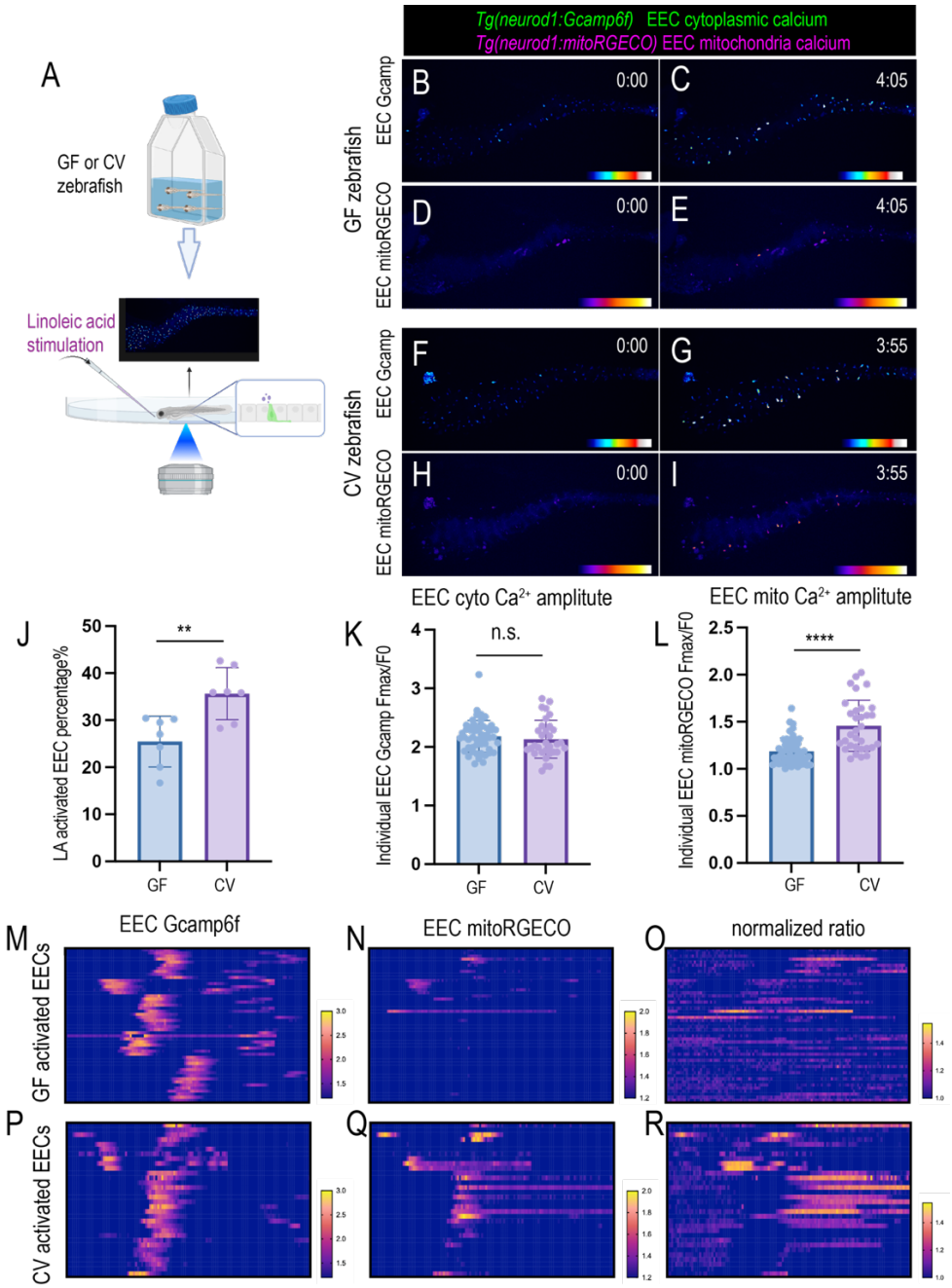


Figure 8. Commensal microbiota alters the EEC cellular and mitochondrial calcium activity in response to nutrient stimulation. (A) In vivo imaging to analyze the 7dpf GF and CV zebrafish EEC cellular and mitochondrial activity in response to linoleic acid stimulation. (B-I) Time-lapse images of the EECs post linoleic acid stimulation in GF and CV zebrafish. The EEC cytoplasmic Ca^{2+} was labeled by Gcamp6f, and the EEC mitochondrial Ca^{2+} was labeled by mitoRGECO. (J) Quantification of the percentage of EECs that are activated by linoleic acid in GF and CV zebrafish. Each dot represents an individual zebrafish. (K-L) Quantification of the linoleic acid-activated EEC cytoplasmic Ca^{2+} amplitudes and the mitochondrial Ca^{2+} amplitudes in GF and CV zebrafish. Each dot represents an individual EEC. More than 4 zebrafish were analyzed in each group.

Note that there is no significant change in the cytoplasmic Ca^{2+} increase. However, CV EECs exhibit a significantly higher mitochondrial Ca^{2+} increase in response to linoleic acid stimulation. (M-R) Analysis of the change of the EEC Gcamp6f fluorescence, EEC mitoRGECO fluorescence, and EEC mitoRGECO/Gcamp6f ratio in GF and CV zebrafish. Only the linoleic acid activated EECs were plotted. M and P, N and Q, O and R use the same heat map scale.

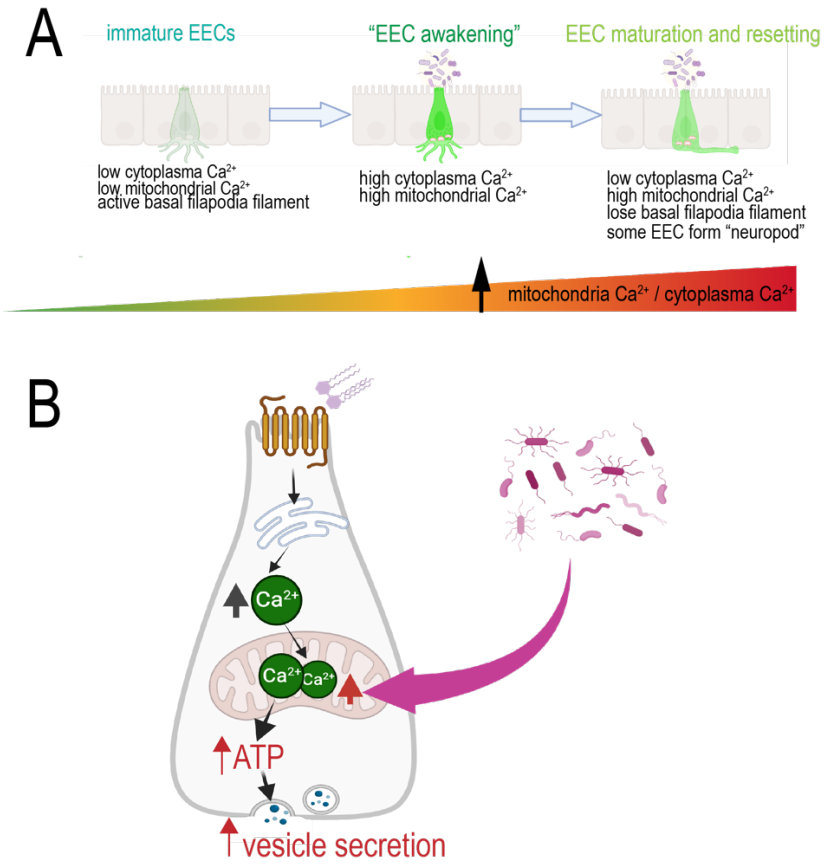


Figure 9. Model figure showing gut microbiota modulate EEC maturation and mitochondrial function. (A) During early development, the immature EECs exhibit low cytoplasmic Ca^{2+} and low mitochondrial Ca^{2+} levels. These immature EECs have active filapodial filaments at the basal lateral membrane. After the zebrafish hatched out and commensal microbiota started to colonize the zebrafish intestine, the EECs continued to develop and mature. Shortly after commensal microbiota colonization, the EECs increase both cytoplasmic and mitochondrial Ca^{2+} significantly ("EEC awakening"). After the EEC awakening, the EECs continue to mature and lose the basal lateral filapodial filamentswhile some EECs form a neuropod. The mature EECs have low cytoplasmic Ca^{2+} but high mitochondria-to-cytoplasm Ca^{2+} ratio. (B) Commensal microbiota promotes EEC mitochondrial respiration function and increases mitochondrial inner membrane electronic potential ($\Delta\Psi m$). When nutrient stimulants, like fatty acids, stimulate the EECs, the EEC cytoplasmic Ca^{2+} rises. The high $\Delta\Psi m$ permits the cytoplasmic Ca2+ to flux into

the mitochondrial matrix and power mitochondrial ATP production, which then promotes EEC vesicle release.

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