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# Interaction between *fam57b* and *cdipt* in a model of 16p11.2 deletion syndrome

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## Nederlands

*Deze masterproef is (ten dele) tot stand gekomen in de periode dat het hoger onderwijs onderhevig was aan een lockdown en beschermende maatregelen ter voorkoming van de verspreiding van het COVID-19 virus. Het proces van opmaak, de verzameling van gegevens, de onderzoeksmethode en/of andere wetenschappelijke werkzaamheden die ermee gepaard gaan, zijn niet altijd op gebruikelijke wijze kunnen verlopen. De lezer dient met deze context rekening te houden bij het lezen van deze masterproef, en eventueel ook indien sommige conclusies zouden worden overgenomen.*

## Engels

*"This master's thesis came about (in part) during the period in which higher education was subjected to a lockdown and protective measures to prevent the spread of the COVID-19 virus. The process of formatting, data collection, the research method and/or other scientific work the thesis involved could therefore not always be carried out in the usual manner. The reader should bear this context in mind when reading this Master's thesis, and also in the event that some conclusions are taken on board".*

Abstract

**Achtergrond**

Het 16p11.2 deletiesyndroom, een neurologische aandoening geassocieerd met verschillende symptomen inclusief leerstoornissen, epilepsie en autisme spectrum stoornis, betreft deletie van een 25 genen kern. Voorafgaande data suggereert dat twee of meer genen bijdragen tot elk symptoom en dat *fam57b*, dat codeert voor een ceramide modulator, één van de belangrijkste genen is in dit syndroom. *cdipt*, dat codeert voor een fosfatidylinositol synthase, is een ander gen in het 16p11.2 deletie-interval dat gelinkt is aan de ceramide pathway. Dit gen interageert met *fam57b*. Daarom stellen we de hypothese voor dat heterozygositeit van zowel *cdipt* als *fam57b* het lipide metabolisme wijzigt in de hersenen wat kan leiden tot neuronale afwijkingen en gedragswijzigingen.

**Doel**

Deze studie beoogt het effect van heterozygositeit van zowel *cdipt* als *fam57b* op de vatbaarheid voor stress en epileptische aanvallen en op het sociaal gedrag in zebravissen te analyseren.

**Resultaten**

De wilde type larven bewogen significant meer dan *fam57ba*$^{+/-}$*cdipt*$^{+/-}$*;fam57bb*$^{+/-}$ en *fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$ larven na blootstelling aan stress (verandering in lichtintensiteit en mechanische storing). De relatieve verandering in afgelegde afstand na het induceren van epileptische aanvallen door middel van de GABA antagonist pentylenetetrazol was hoger in zowel *fam57ba*$^{+/-}$*cdipt*$^{+/-}$*;fam57bb*$^{+/-}$ als *fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$ larven vergeleken met wilde types. Larven van deze genotypen zijn dus vatbaarder voor epileptische aanvallen. Er werden geen significante verschillen gezien in sociaal gedrag tussen de verschillende genotypes.

**Conclusie**

Heterozygositeit van zowel *cdipt* als *fam57b* beïnvloedt de stress respons en vatbaarheid voor epileptische aanvallen in zebravissen. Dit kan duiden op de betrokkenheid van GABA-ergische inhibitorische neuronale activiteit in het ontstaan van symptomen van het 16p11.2 deletiesyndroom.

Abstract

**Background**

16p11.2 deletion (16pdel) syndrome is a neurodevelopmental disorder associated with symptoms including learning disability, epilepsy and autism spectrum disorder. A core of 25 encoded genes is deleted in this syndrome. Previous data suggests that two or more genes contribute to each symptom, and that *fam57b*, that encodes a putative ceramide modulator, is a key gene in this syndrome. *cdipt* encodes a phosphatidylinositol synthase and is another gene in the 16pdel interval. It is connected to the ceramide pathway and interacts genetically with *fam57b*. Therefore, we hypothesize that combined heterozygosity of *fam57b* and *cdipt* affects lipid metabolism in the brain, possibly contributing to neuronal anomalies and changes in behavior.

**Aim**

The aim of this study was to assess the effect of combined heterozygosity of *fam57b* and *cdipt* on stress, seizure susceptibility and social behavior.

**Results**

Wild type larvae moved significantly more than both *fam57ba*$^{+/-}$*cdipt*$^{+/-}$*;fam57bb*$^{+/-}$ and *fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$ larvae after being subjected to a stressor (changes in light intensity and mechanical disturbance) . The relative change in distance moved post-induction of seizures with the GABA antagonist pentylenetetrazol was higher in both *fam57ba*$^{+/-}$*cdipt*$^{+/-}$*;fam57bb*$^{+/-}$ and *fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$ larvae compared to wild type. This indicates that *fam57ba*$^{+/-}$*cdipt*$^{+/-}$*;fam57bb*$^{+/-}$ and *fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$ larvae are more susceptible to seizures. No significant difference in social behavior was detected between genotypes.

**Conclusion**

Combined heterozygosity of *cdipt* and *fam57b* influences the stress response and seizure susceptibility of zebrafish. The data may suggest contribution of GABAergic inhibitory neuronal activity to symptoms of 16p11.2 deletion syndrome.

Introduction

Neurodevelopmental disorders have a high prevalence, affecting approximately 20% of the US population[1]. Most of these disorders only have a limited number of treatment options. This is mainly due to a poor understanding of the mechanisms involved in these diseases. 16p11.2 deletion (16pdel) syndrome is an insufficiently understood neurodevelopmental disorder. It comprises a broad range of phenotypes, and is tightly associated with neurodevelopmental disorders, including autism spectrum disorder, intellectual disability, seizures, and Attention-deficit/Hyperactivity disorder[2]. It is estimated to affect 1 in 2,000 individuals[3]. Approximately 600kb, including a core of 25 encoded genes, are deleted in one copy of chromosome 16 in patients with this disorder. The functions of most of these genes are unclear and thus the role they play in this syndrome remains elusive[4]. Understanding the pathways and mechanisms involved in 16pdel syndrome can help us find new targets for therapy.

One of the genes included in the deletion is *fam57b.* After loss of one copy, *fam57b* showed genetic interaction with a significant number of other genes included in the 16p11.2 interval and is thus considered a key hub gene in the syndrome[5]. The encoded protein is connected to the ceramide pathway through its function as a putative ceramide modulator[6,7] (unpublished data from the Sive lab). In general, lipids play a role in synaptogenesis, neurogenesis and in the development and maintenance of the brain[8,9]. Ceramides in particular have a variety of physiological functions, including intracellular signaling[10] and serve as a building block for sphingomyelin synthesis[11]. They are also important membrane components[12].

It has previously been described that several genes, at least 2 or more, in the 16p11.2 interval work together through indirect mechanisms to mediate different phenotypes of the syndrome[5]. *cdipt,* another gene in the interval, was found to genetically interact with *fam57b*[5]. The encoded protein, a CDP-diacylglycerol-inositol 3-phosphatidyltransferase, is also connected to the ceramide pathway. *cdipt* is the only phosphatidylinositol synthase found in zebrafish[13], and complete loss-of-function is embryonic lethal. Phosphatidylinositol is also an important component of the plasma membrane. It can be converted into phosphatidylcholine that is necessary for sphingomyelin synthesis[14]. When genes interact it means that the phenotype resulting from the product of those genes cannot be predicted by the phenotype resulting from either of these gene products alone[15]. Since these two genes, *fam57b* and *cdipt* interact and both play a role in the ceramide pathway and in lipid synthesis in general, it is of high interest to study their combined effect. **We hypothesize that combined heterozygosity of *cdipt* and *fam57b* alters the lipidomic profile in zebrafish brains, possibly contributing to neuronal anomalies and changes in downstream function.** Our primary goal is thus to analyze the effect of combined heterozygosity of *cdipt* and *fam57b* on the behavior of zebrafish larvae.

The zebrafish are an excellent system to study neurodevelopmental disorders due to several factors. The ease of genetic manipulation of zebrafish, their quick and ample reproduction as well as their rapid development make zebrafish the model of choice to analyze this hypothesis. They are genetically and phenotypically highly homologous to humans, however for some genes a second copy is present in the zebrafish[16]. This is the case for *fam57b*, as there are two copies of this gene in zebrafish, *fam57ba* and *fam57b.* Both *fam57ba* and *cdipt* are located on chromosome 3 whereas *fam57bb* is located on chromosome 12[17].

Results

## *fam57ba*$^{+/-}$*cdipt*$^{+/-}$*;fam57bb*$^{+/-}$ and *fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$ larvae display differential responses to stress compared to wild type larvae

Motor impairments, hypotonia and movement disorders have previously been associated with 16pdel syndrome[18,19]. By recording the baseline movement of larvae (fig. 1A), it is possible to look at differences between the genotypes of interest. However, no significant difference in baseline movement has been seen between the tested genotypes and the wild type (AB) larvae (fig. 1B). Since the behavioral assays used to look at the downstream effects of combined heterozygosity of *fam57b* and *cdipt* highly rely on the movement of the larvae, it was important to see if there were significant differences in baseline movement. A difference in motility of the fish could result in a bias in the subsequent behavioral tests.

It has been shown that 16pdel mice respond differently to stress compared to wild type mice, they tend to display a more active coping strategy[20]. Anxiety disorders and differences in assessment of stressful situations have also been linked to autism spectrum disorder[21-23]. Therefore, we hypothesize that combined heterozygosity of *cdipt* and *fam57b* results in an altered stress response. This can be assessed during the seizure assay. After a habituation period of 10 min and a baseline recording of 10 min in the dark recording chamber of the Noldus Daniovision, the plate containing the larvae is removed and the larvae are again exposed to the intense light of the laboratory environment. Changes in illumination, particularly from dark to light, have been shown to induce stress in larvae. Furthermore, the duration of the exposure to light has an effect: the longer the light exposure, the higher the locomotor activity in larvae[24]. Changes in heat, UV exposure and mechanical disturbance have also been associated with stress responses and higher cortisol levels in zebrafish larvae[25]. There are still uncertainties and areas to explore when it comes to stressors for zebrafish. In this experimental setting (fig. 1A), the light intensity is not the only thing that changes during the seizure assay, 100 µl of E3 (this is half the volume in which they swim) is removed by pipetting in the well containing the larvae whereafter 100 µl of E3 is added again. This could be compared to mechanical disturbance and considered as a potential stressor for zebrafish larvae. The plate containing the larvae is then placed again in the dark recording chamber for a subsequent 10 min recording. Stress can generate different types of behaviors in zebrafish, it can induce freezing, which would show a decrease in movement compared to baseline, or it can induce erratic movements which would show an increase in movement compared to baseline[26]. The AB larvae show a significant increase in movement ($P < 0.0001$) after the stressing events, however the other genotypes do not display any significant difference compared to baseline (fig. 1B). The distance moved after the stressors is significantly higher in AB's compared to *fam57ba*$^{+/-}$*cdipt*$^{+/-}$*;fam57bb*$^{+/-}$ ($P = 0.0003$) and *fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$ ($P = 0.0008$). This would indicate that heterozygosity for *fam57b* and combined heterozygosity of *fam57b* and *cdipt* can lead to a difference in stress response in zebrafish.

## *fam57ba*$^{+/-}$*cdipt*$^{+/-}$*;fam57bb*$^{+/-}$ and *fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$ larvae show increased seizure propensity

Individuals with 16pdel syndrome are strongly associated with increased seizure frequency as a recurrent symptom[27,28]. Baraban *et al.* have observed seizures in zebrafish, as defined by behavioral and electrophysiological changes, that closely resemble those observed in rodents and humans. This study identified 7 days post fertilization (dpf) zebrafish larvae as an optimal developmental time to study chemically induced seizures. They showed that pentylenetetrazol (PTZ), a noncompetitive GABA-A antagonist, can be bath-applied in small volumes to induce

seizures in zebrafish larvae[29]. Increased swimming speed, and thus distance moved, is directly correlated with the epileptiform discharges present in seizure electrophysiology[5]. Therefore, Berghmans *et al.* developed a high-throughput behavioral approach to study seizure propensity in zebrafish by measuring distance moved after acute PTZ treatment[30]. We adapted this protocol from Berghmans *et al*. (fig. 1A). A significant increase in activity after PTZ treatment was detected in all four genotypes (respective P values: P < 0.0001 for AB, *fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$ and *fam57ba*$^{+/-}$*cdipt*$^{+/-}$*;fam57bb*$^{+/-}$, and P = 0.0009 for *cdipt*$^{+/-}$) showing that the PTZ treatment at 5 mM was effective. The distance moved after PTZ treatment is significantly higher in *fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$ larvae compared to AB controls (P = 0.0147). Although the difference between AB and *fam57ba*$^{+/-}$*cdipt*$^{+/-}$*;fam57bb*$^{+/-}$ is not statistically significant, the same trend is followed. When looking at the relative fold changes, the increase in distance traveled is much greater for *fam57ba*$^{+/-}$*cdipt*$^{+/-}$*;fam57bb*$^{+/-}$ (fold change = 13.78) and *fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$ larvae (fold change = 11.60) than for AB controls (fold change = 2.92). This indicates that heterozygosity of *fam57b* and combined heterozygosity of *fam57b* and *cdipt* leads to a higher seizure susceptibility.

**Heterozygosity of *fam57b* and *cdipt* does not significantly alter social preference in zebrafish larvae**

As previously mentioned, 16pdel syndrome has been associated with autism spectrum disorder and Attention-deficit/Hyperactivity disorder, among other symptoms[2]. Social difficulties are often if not always associated with these disorders[31,32]. Since altered social behavior is a contributing factor in some phenotypes caused by 16pdel syndrome, we hypothesized that social preference would be altered by combined heterozygosity of *fam57b* and *cdipt*. Zebrafish are gaining popularity for studying several complex brain disorders, including disorders affecting social behaviors[33]. Varying assays have been developed to study social preference of zebrafish at different ages[34-37]. We based our analysis on the three-chamber model developed by Dreosti *et al.* to study the evolution of social behavior in developing zebrafish. They concluded that social behavior, including social preference and social avoidance, increases with age and is robust at 21 dpf, which is the timepoint we chose for this analysis[34]. The dimensions and layout of the chamber used for the social assay are represented in fig. 2A. The left and right side of the test chamber are identical; therefore, we can assume that there should not be a preference for a specific side based on our experimental setup. Since the plate used for the social assay was custom made, the plate was disinfected with ethanol between repeats of the experiment. Ethanol is known to influence locomotion, anxiety, aggression, shoaling and preference for social novelty in zebrafish[36,38,39]. We therefore thoroughly rinsed the plate with de-ionized water to negate the effects of ethanol on behavior.

As defined by Dreosti *et al.* preference for a chamber is calculated in the absence and presence of social cues (SCs) (fig. 2B-C), whereafter it is assessed if there is an increase in preference for the side where the SCs (two age matched AB controls) are added[34]. The recordings were done in the Noldus Daniovision recording chamber. This device is coupled to the Ethovision XT software for animal tracking. However, for this assay some issues were detected with the software. It only tracked moving larvae, therefore, the time a larva stays immobile in a chamber is not factored in the results, this yields a bias in our analysis. No significant differences were found in social preference across genotypes (fig. 2D).

When comparing these results with the individual traces of the fish, some issues with the social behavior analysis came to light. In this scenario, if a larva has a strong preference for the side where the SCs will be added (future SC chamber) and that larva still displays a preference for

that side once the SCs are added (SC chamber), there will be no increase in preference for the SC side. This larva will therefore be classified as socially neutral. If a larva does not observe the social cues (it starts by the empty chamber), it cannot display social preference or avoidance. For example, one fish can swim a few minutes unknowingly by the empty chamber, after a while it discovers there is a passage from that side of the test chamber to the SC side. Once it observes the SCs, it exhibits social preference until the end of the recording. The calculated social preference of that fish will be lower compared to a fish that observed the social cue earlier and exhibited social preference. Therefore, not factoring in the latency to discover the SCs gives a bias in interpretation of the social behavior of that fish. The data obtained from the Ethovision XT software does not give the appropriate data in order to factor in the latency to discover the SCs. Therefore, the movies obtained from the recording with social cues were manually scored using a software called BORIS[40]. The manual scoring of the video's was compared to the scoring based on the Ethovision XT software. No significant differences in score was observed (Supplementary fig. 1) indicating that the results obtained by manual scoring are precise enough to derive conclusions from them.

The latency to discover the SCs is the amount of time the fish spends by the empty chamber between the onset of the recording and the first time it crosses to the SC chamber. To factor in the latency to discover the SCs, I created the normalized social preference formula (fig. 2C). By analyzing the manually scored data with the new formula, the small differences and large spread of data seen in fig. 2D, were reduced. No significant differences were detected (fig. 2E). The distribution of the data seems to be very similar between the different genotypes. The larvae heterozygote for *cdipt* show a small trend towards more neutral or even avoidance behavior, however the sample size for this genotype was very small. When factoring in the latency to discover the SCs, the larvae that do not explore the SC chamber are automatically excluded. Exploratory behavior has been linked to stress coping, fish that explore more are thought to have a more proactive coping mechanism[41]. Therefore, keeping track of how many fish never explored the SC chamber is of interest. A total of 3.45% of the AB's never explored the SC chamber, this is much lower than the other genotypes (17% for *fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$, 10.71% for *fam57ba*$^{+/-}$*cdipt*$^{+/-}$*;fam57bb*$^{+/-}$ and 22.2% for *cdipt*$^{+/-}$). This suggests that the genetic lines we tested have an altered stress coping mechanism compared to AB controls.

Discussion

Connecting multigenic disorders to multiple associated symptoms still remains challenging. The deletion of a core of 25 genes in 16p11.2 deletion syndrome is linked to a variety of phenotypes[2]. However, the role of these genes in pathophysiology is still poorly understood. Previously, it has been described that the interaction of multiple genes is responsible for the mediation of different phenotypes included in this syndrome[5]. The purpose of this study was to shed some light on the combined effect of two genes included in this syndrome, *cdipt* and *fam57b*. Multiple conclusions have been reached: heterozygosity of *fam57b* and combined heterozygosity of *cdipt* and *fam57b* in zebrafish results in a higher seizure susceptibility, suggests an altered response to stress, but does not seem to significantly alter social preference.

The results obtained by looking at the stress response and at the social behavior would indicate that heterozygosity of *fam57b* and combined heterozygosity of *cdipt* and *fam57b* influences the response to stress and the stress coping mechanisms. The small number of repeats for zebrafish heterozygous for *cdipt* does not allow us to make strong conclusions concerning this genotype. More robust assays to look at stress have been developed, including thigmotaxis, dark-startle response and cortisol levels, they could provide more precise information on the effect of (combined) heterozygosity of *cdipt* and *fam57b* in zebrafish[42,43]. Looking at exploratory

behavior in a more adapted setup and for a longer period of time would provide a more objective view of the stress coping mechanisms from the different genetic lines[41]. Several pathways have been linked to stress response in zebrafish by analyzing the effects of pharmacological treatments on behavior[44]. Recently a study linking GABAergic activity to stress response, showed that stimulation of GABAergic neurons reduces anxiety-like behavior and inhibition of GABA receptors enhances anxiety-like behavior[45]. This may indicate that *fam57b* and/or *cdipt* influence GABAergic inhibitory activity.

Seizures have previously been linked to an imbalance between excitatory and inhibitory neuronal activity[46]. To induce the seizures in our seizure assay, pentylenetetrazol (PTZ) was used, this drug acts through inhibition of GABAergic inhibitory activity[47]. The results obtained through the seizure assay indicates that heterozygosity of *fam57b* and combined heterozygosity of *cdipt* and *fam57b* induces a substantial increase in seizure susceptibility. Since the seizures are induced by PTZ, it can be deduced that those genetic lines are subjected to an overactivity of GABAergic inhibitory circuits. This overactivity could possibly explain the differences in stress responses mentioned above. It would be interesting to take a closer look at GABAergic inhibitory activity as well as glutamatergic excitatory activity, in other words at the balance between excitation and inhibition in relation to *fam57b* and *cdipt*. The imbalance between those two types of neuronal activity can be caused by several factors. Anomalous synaptic transmission and aberrant synaptic proteins have been linked to imbalances in excitation and inhibition as well as seizure activity[46,48]. Previously in our lab the lipidomic profile of *fam57b* in heterozygotic and double homozygotic larvae has been analyzed. There were significant alterations in lipid species present in the brain, more precisely in lipid species that are essential components of the plasma membrane and that are related to the ceramide pathway (unpublished data). Our lab also showed that loss of function of *fam57b* resulted in an anomalous plasma membrane, mislocalization of presynaptic proteins and abnormal neuronal activity (unpublished data). Based on all this information, we hypothesize that (combined) heterozygosity of *fam57b* and *cdipt* leads to an altered lipidomic membrane architecture, possibly at the level of the synapse, which could lead to abnormal membrane protein recruitment. This can lead to anomalous synaptic function and thus to abnormal firing patterns in the brain which would ultimately result in abnormal downstream function. In this study we showed that the downstream function is indeed altered. However, further analysis is needed to support this hypothesis. Analyzing the lipidomic and proteomic profile, along with electrophysiology of the brain of larvae across genotypes will enable us to test our hypothesis. However, before being able to perform those analyses, we first had to find a technique to genotype larvae at an early stage prospectively since retrospective analysis is not possible in these cases. Therefore, three options were tested and described in the supplementary methods.

A high throughput technique for zebrafish larvae genotyping, called the Zebrafish Embryonic Genotyper (ZEG), was developed by C. Lambert *et al.*[49]. This device works by placing a dechorionated zebrafish embryo or larvae on oscillating roughened glass. The overall survival rate was low (37.5% survival after 24 h; n = 32), and the PCR results were of poor quality. Changing the voltage of the device from 1.4 V to 0.7 V to lower the oscillation rate, did not improve the survival rate. The PCR quality was slightly improved by adding a short lysis step, however, the survival rate was too low to pursue this option. A genotyping technique based on environmental DNA (eDNA) of 3-5 days post hatch sheepshead minnow was developed by Espinoza *et al.*[50]. This is a noninvasive technique that would minimally influence the development of the larvae. To reduce the effect of social isolation on development, we tried this method on 1-3 dpf zebrafish larvae. The PCR results seemed to indicate contamination issues. The results obtained through eDNA did not correspond to the results obtained after

sacrificing the larvae. This technique appeared promising, however there were sensitivity issues that we were unable to resolve. Therefore, we chose to test a third method by genotyping 3 dpf zebrafish larvae by taking a tail biopsy [51,52]. The overall survival rate was 96.9% (n = 32) and no contamination was detected. When viewing the tail tissue under a microscope at 7 dpf, there seemed to be a good recovery (Supplementary fig. 2). However, for a minority of larvae the tail grew back crooked, this could be dependent on the angle at which the tail is cut. Since the survival rate, PCR results and recovery from the biopsy were promising, this technique can be used to genotype the larvae destined for the lipidomic and electrophysiologic analyses.

Based on our results, no difference in social preference was detected. However, compared to the study performed by Dreosti *et al.* our n values for assessing social behavior are relatively small[34]. Social behavior is very complex and can be influenced by many factors. A higher n value can lead to a clearer view of the social tendencies associated with different genotypes. Social behavior is assayed in a large variety of ways in the literature[35-37]. Every setup and analysis differs, and it is thus possible that another experimental setup might lead to different results. Since Dreosti *et al.* did not take the latency to discover the SCs into account, their results may be biased, so it is possible that 21 dpf is too early to assess differences in social preference, other assays also use later timepoints[34-37]. Studying the ontogeny of social behavior would enable us to determine the optimal window to study social behavior. There is no clear consensus on what social behavior is, defining how close the larvae need to be to the SCs for their behavior to actually be considered as social would improve the way social behavior is assessed at this time. Dreosti *et al.* showed that social behavior in zebrafish larvae mainly depends on visual cues[34], but no information is given on visual capacity of the larva. Determining that distance and including that parameter in the design of social assays would improve the way we assay social behavior. The manual scoring of the videos is a tedious and time-consuming but necessary process, and developing a software for this analysis is necessary. As mentioned before, social behavior is complex, it is possible that our experimental setup lacks sophistication to fully grasp fish behavior. By separating the test larvae from the social cues with a transparent divider, some typical social interactions cannot be assessed. Wild type zebrafish have a natural tendency to display schooling and shoaling behavior[26]. These social behaviors could be assessed in a more naturalistic setup through machine learning and 3D tracking of fish. The pathways involved in the creation of social behavior are still poorly understood, a better insight in those are needed to draw conclusions from such assays.

Overall in this study we show that (combined) heterozygosity of *fam57b* and *cdipt* influences several factors such as response to stress and seizure susceptibility. The differences in phenotype between heterozygosity of *fam57b* and combined heterozygosity of *fam57b* and *cdipt* do not seem significant, this would suggest that *fam57b* plays the major role in mediating those phenotypes. However, the relative fold change for the *fam57ba*$^{+/-}$*cdipt*$^{+/-}$*;fam57bb*$^{+/-}$ larvae is higher than for the *fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$ larvae in the seizure assay, this would suggest that combined heterozygosity of *fam57b* and *cdipt* results in an additive effect. More repeats are necessary to explore this possibility as well as to assess if the phenotype caused by heterozygosity of *cdipt* is significantly different from the other genotypes. Since 16pdel syndrome is a developmental disorder, it would be interesting to look at all these parameters from embryos to adults to get a more complete view of this disorder and the influence of these two genes.

Methods

**Fish lines and husbandry**

Adult zebrafish were housed in 2L tanks, with a maximum of 4 fish per tank, maintained at optimal conditions (pH: 6.5-7.5, temperature: 26.5-28 °C) on a 12 h/12 h light/dark cycle. Embryos were obtained through natural spawning and raised in daily renewed embryo medium (E3) at 28-28.5 °C. 1% Methyl blue, an antifungal agent, was added to the embryo medium for the first 72hpf. E3 is composed of 4.96 mM NaCl, 174.38 mM KCl, 333.39 mM $CaCl_2$ and 162.29 mM $MgSO_4$ x $7H_2O$. When the larvae reach 7 dpf they are fed paramecia. Triple (*fam57ba*$^{+/-}$*cidpt*$^{+/-}$*;fam57bb*$^{+/-}$) and double (*fam57ba*$^{+/-}$*;fam57bb*$^{+/-}$) heterozygous fish were obtained by crossing double homozygote fish for *fam57ba* and *fam57bb* to heterozygous fish for *cdipt.* Whilst wild type fish were obtained by crossing AB fish.

**Genotype identification**

For the behavior assays, the genotypes of the larvae were identified retrospectively by sacrificing the larvae and lysing the whole larvae with proteinase K. The larvae were genotyped for *cdipt* using the following primers: forward primer (F): CCCACACGTTTAATAGGATGTGC and reverse primer (R): TTTGTACCTAAGGACGAGGCAC. After restriction digestion with the enzyme ApaLI a product of 238 bp was observed for the wild type larvae and products of the following sizes were observed for heterozygous fish: 238, 206 and 19 bp. The primers for *fam57ba* were: F: TTGGTTTCAAGTGTCTCCTGTC and R: TCGCACTCCTACACATCTGG, those for fam57b were: F: GAGATGAACCGATTGTCAACAT and R: GTACCTAATAAAGTGGCCGGTG. The respective enzymes were BtgZ1 for *fam57ba* resulting in products of 236 and 94 bp, and Ear1 for *fam57bb* resulting in products of 270 and 127 bp for the heterozygous fish.

**Behavior assays**

Seizure assay

Larvae (7 dpf) were individually pipetted with 200 µl of E3 in flat bottom 96-well plates. They were then transferred to the Noldus Daniovision observation chamber for a 10min habituation period. This was followed by 10 min of baseline movement recording. 100 µl of E3 was then removed from each well and replaced by either 100 µl of E3 (column 1-6) or 100 µl of concentrated pentylenetetrazol (PTZ) solution (column 7-12), bringing the PTZ concentration in those wells to 5 mM. This was immediately followed by another 10 min recording. The distance moved was then calculated using the Ethovision XT software. The baseline movement is assessed by looking to the first 6 columns of the first recording. When the plate is removed from the recording chamber, the fish are subjected to a change in light intensity. This, and the removal and consecutive addition of 100 µl of E3 can be stressful for the larvae. Therefore, by comparing the amount of movement in the first and second recording for the first 6 columns, the influence of stress on the larvae can be assessed. The baseline movement was subtracted from the movement in the second recording, to normalize the obtained results before looking at the seizure susceptibility. A schematic overview of this assay can be found in fig. 1A.

Social preference assay

A fish (21dpf) was placed in a custom 3D printed behavioral setup (fig. 1A) filled with 5ml of E3. Two chambers are separated by glass dividers from the area where the test fish can swim freely. The system was placed in the Noldus Daniovision observation chamber with a 10% light intensity and a recording of 15 min was done by the Ethovision XT software, to assess

the baseline movement and if there is a preference for a side. After the baseline recording, two age-matched AB fish are placed in either the left or right separate chamber, these serve as social cue (SC). The movement of the test subject is then recorded for 15 min. The movies were manually scored using the BORIS software[40]. The baseline recording is used to determine if the larvae has a preference for a chamber. This is assessed by calculating the chamber preference index (CPI) according to the following formula: CPI = (time spent by the empty chamber - the time spent by the chamber where the SC will be added)/ total time. The datapoints with less than 800 s as cumulative duration were excluded from this analysis. This results in a value ranging from -1 to 1. The social preference index (SPI) is then calculated based on the recording with SC according to the following formula: SPI = (time spent by SC – time spent away from SC) / total time. The SPI ranges from -1 (strong social aversion) to +1 (strong social preference). The normalized SPI is calculated with the following formula: (time spent by SC – (time spent away from SC – latency to see SC)) / (total time – latency to see SC).

**Statistical analysis**

The seizure assay (fig. 1C) and social preference assay (fig. 2D-E) were analyzed for normality with the Shapiro-Wilk test. They did not meet the assumption of normality and were therefore assayed using a Kruskal-Wallis test for multiple comparisons. The data for the response to stress (fig. 1B) was analyzed for multiple comparisons using a two-way ANOVA since we want to see differences between baseline and stressor for each genotype as well as the differences between the different genotypes for each parameter (baseline and stressor). The n values, means and standard errors are given in tables under the respective graphs and the P values are given in each legend. All the statistical tests were done using Graphpad Prism version 8.4.2. for MacOs (GraphPad Software Inc., La Jolla California USA, www.graphpad.com).

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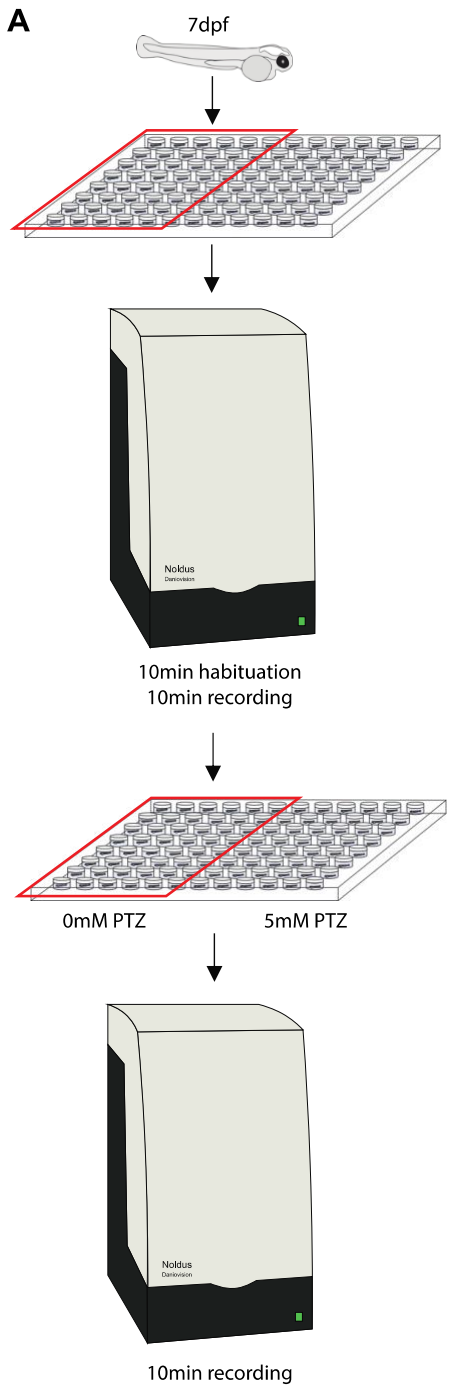
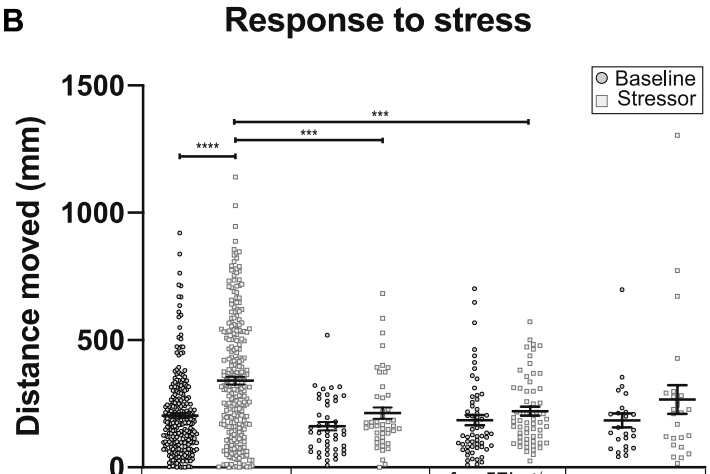
Figure legends

**Figure 1: Combined heterozygosity of *fam57b* and *cdipt* alters the seizure propensity and response to stress.** A) Schematic overview of the experimental setup. The red box indicates the wells that do not receive PTZ treatment, these are the data points used for the analysis of the response to stress. B) The distribution of the total distance moved shows that the increase in movement, after being subjected to stress, is significantly higher for AB larvae compared to *fam57ba*$^{+/-}$,*cdipt*$^{+/-}$;*fam57bb*$^{+/-}$ and for *fam57ba*$^{+/-}$;*fam57bb*$^{+/-}$ larvae. (****P < 0.0001, ***P = 0.0008, ***P = 0.0003). C) The distance moved increased significantly after PTZ treatment for all genotypes (****P < 0.0001, ***P = 0.0009, *P = 0.0147). The relative fold changes indicate that the increase in distance moved is much higher for *fam57ba*$^{+/-}$,*cdipt*$^{+/-}$;*fam57bb*$^{+/-}$ and for *fam57ba*$^{+/-}$;*fam57bb*$^{+/-}$ larvae compared to AB and *cdipt*$^{+/-}$ larvae. The n values, mean, standard error and relative fold change are stated under each graph.

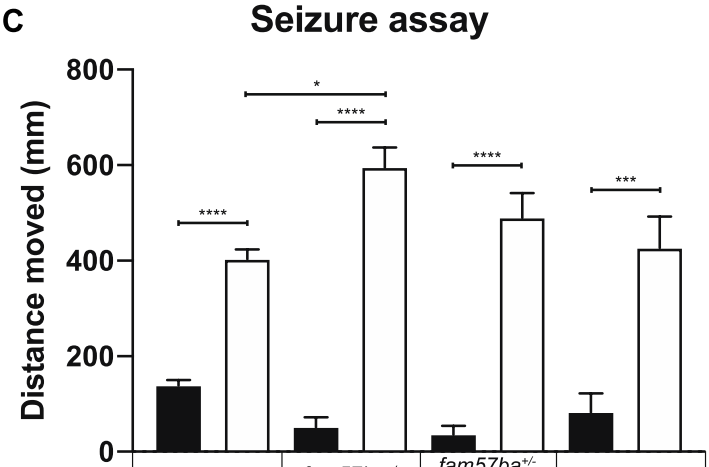
**Figure 2: The effect of combined heterozygosity of *fam57b* and *cdipt* on social behavior in zebrafish larvae.** A) Representation of the social behavior chamber in 3D including the dimensions. The grey dividers represent transparent glass dividers that allow flow of water but do not allow zebrafish larvae to cross. B) Schematic representation of the social assay. The social cue (SC) larvae are added to the left SC chambers for the two left testing chambers and to the right SC chambers for the two right testing chambers. C) The first formula was used to calculate the data used in panel D; the second formula was used to calculate the data used in panel E. D) Social preference assessed by calculating the chamber preference of the baseline recording compared to the recording with added SCs. Look at the increase in preference for the side with the social cues. This assay did not yield significant differences. E) Social preference assessed by factoring in the latency to discover the SCs this leads to the normalized social preference index. There are no significant differences between the different genotypes. The normalized social preference index automatically excludes larvae that did not explore the SC chamber (data excluded: 2/58 AB, 8/47 for *fam57ba*$^{+/-}$; *fam57bb*$^{+/-}$, 3/28 for *fam57ba*$^{+/-}$,*cdipt*$^{+/-}$;*fam57bb*$^{+/-}$ and 2/9 for *cdipt*$^{+/-}$).

**Figure 1**



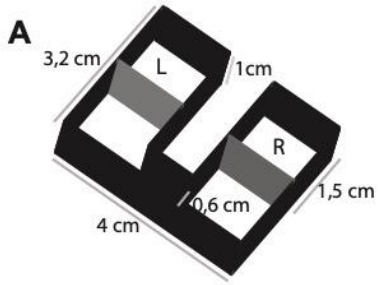



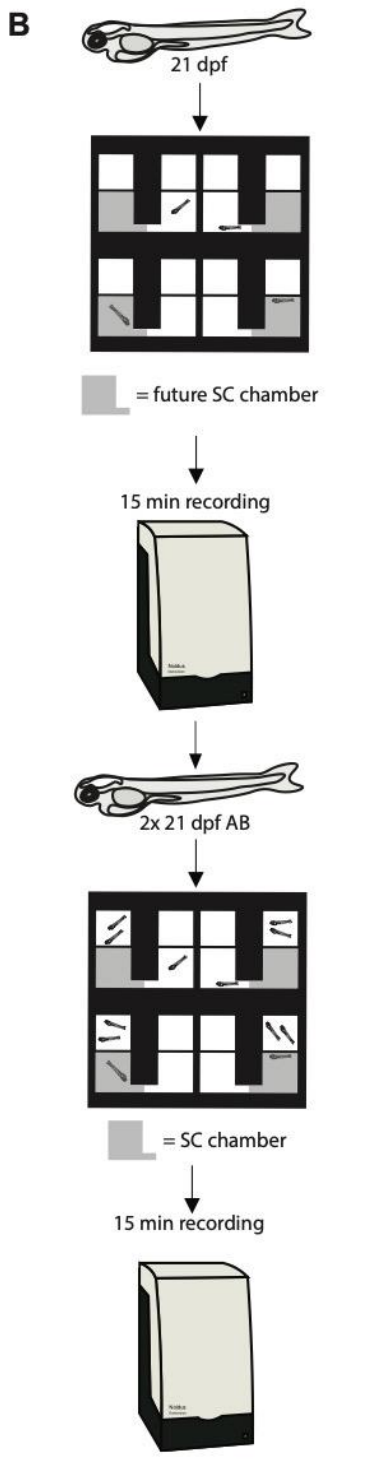

| | AB | | *fam57ba*$^{+/-}$ *fam57bb*$^{+/-}$ | | *fam57ba*$^{+/-}$ *cdipt*$^{+/-}$ *fam57bb*$^{+/-}$ | | *cdipt*$^{+/-}$ | |
|---|---|---|---|---|---|---|---|---|
| n value | 256 | | 45 | | 60 | | 25 | |
| mean | 202.89 | 340.96 | 161.80 | 213.10 | 185.26 | 220.78 | 184.37 | 266.92 |
| std error | ±9.98 | ±14.88 | ±16.10 | ±22.62 | ±19.75 | ±17.34 | ±27.90 | ±56.41 |
| fold change | 1.68 | | 1.32 | | 1.19 | | 1.45 | |




| | AB | | *fam57ba*$^{+/-}$ *fam57bb*$^{+/-}$ | | *fam57ba*$^{+/-}$ *cdipt*$^{+/-}$ *fam57bb*$^{+/-}$ | | *cdipt*$^{+/-}$ | |
|---|---|---|---|---|---|---|---|---|
| PTZ | 0mM | 5mM | 0mM | 5mM | 0mM | 5mM | 0mM | 5mM |
| n value | 256 | 263 | 45 | 48 | 60 | 42 | 25 | 15 |
| mean | 138.06 | 403.09 | 51.30 | 595.24 | 35.52 | 489.56 | 82.55 | 426.53 |
| std error | ±12.29 | ±20.76 | ±21.13 | ±42.04 | ±18.39 | ±52.28 | ±39.55 | ±65.84 |
| fold change | 2.92 | | 11.60 | | 13.78 | | 5.17 | |

**Figure 2**



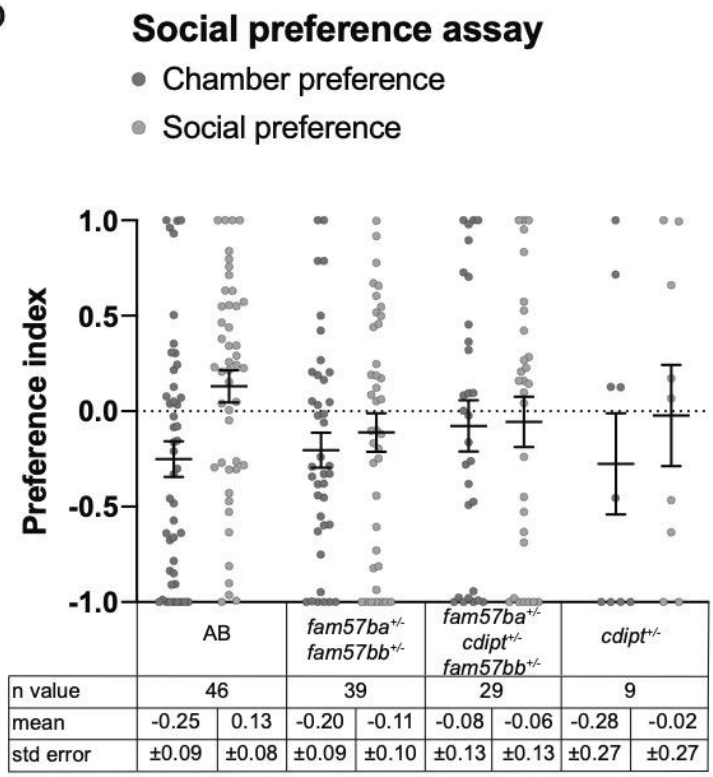




C

$$\text{Preference index} = \frac{t_{\text{(future) SC chamber}} - t_{\text{other chamber}}}{t_{\text{total}}}$$

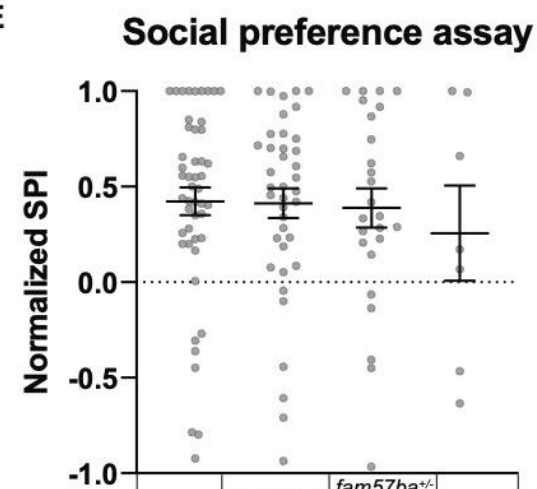
$$\text{Normalized SPI} = \frac{t_{\text{SC chamber}} - (t_{\text{other chamber}} - t_{\text{latency to see SC}})}{t_{\text{total}} - t_{\text{latency to see SC}}}$$

D




| | AB | | *fam57ba*$^{+/-}$ *fam57bb*$^{+/-}$ | | *fam57ba*$^{+/-}$ *cdipt*$^{+/-}$ *fam57bb*$^{+/-}$ | | *cdipt*$^{+/-}$ | |
|---|---|---|---|---|---|---|---|---|
| n value | 46 | | 39 | | 29 | | 9 | |
| mean | -0.25 | 0.13 | -0.20 | -0.11 | -0.08 | -0.06 | -0.28 | -0.02 |
| std error | ±0.09 | ±0.08 | ±0.09 | ±0.10 | ±0.13 | ±0.13 | ±0.27 | ±0.27 |

E




| | AB | *fam57ba*$^{+/-}$ *fam57bb*$^{+/-}$ | *fam57ba*$^{+/-}$ *cdipt*$^{+/-}$ *fam57bb*$^{+/-}$ | *cdipt*$^{+/-}$ |
|---|---|---|---|---|
| n value | 48 | 39 | 25 | 7 |
| mean | 0.42 | 0.41 | 0.39 | 0.26 |
| std error | ±0.07 | ±0.08 | ±0.10 | ±0.25 |

**Supplementary information**

# Interaction between *fam57b* and *cdipt* in a model of 16p11.2 deletion syndrome

Impact of COVID-19

The closing of the research institute in early March due to COVID-19, resulted in the loss of data. The samples for lipidomic analysis were collected, but could not be processed. The training process to enable the acquisition of electrophysiological data from live zebrafish larvae was completed, but the actual analysis on the genotype of interest could not be done. Repeats of the seizure and social assays were planned (especially for the completion of *cdipt*$^{+/-}$ dataset), but could not be executed. This additional data would have allowed for a more complete view of the interaction between *fam57b* and *cdipt*.

Methods

## Genotyping techniques

Zebrafish embryonic genotyper (ZEG)

The Zebrafish Embryonic Genotyper (ZEG) works by placing a zebrafish embryo or larvae on oscillating roughened glass. We tested this device according to the protocol by C. Lambert et al.[1] on 1dpf embryos (n=8), 2dpf embryos (n=8) and 3dpf larvae (n=16). However, we used the standard protocol from our lab for the PCR analysis (Supplementary figure 2B). For a second test the voltage of the device was changed from 1.4V to 0.7V and a short lysis step (30 min of incubation with 2µl lysis buffer and 0,25µl Proteinase K) was added.

Environmental DNA

This method was tested on 1-3 dpf zebrafish larvae. The embryos were isolated around 6 hpf by following the wash steps described in the protocol by Espinoza *et al.*[2], until they hatched naturally. In subsequent tests, dechorionated or hatched larvae (1-3 dpf) were isolated for overnight incubation. Two more wash steps were added in larger volumes of E3. After incubation the supernatant was used to isolate environmental DNA (eDNA) with the Zymo Quick-DNA Miniprep Kit. The standard protocol from our lab was then followed for PCR analysis (Supplementary figure 2B). To detect contamination issues, the larvae were sacrificed, lysed using proteinase K and genotyped according to the same protocol.

Tail biopsy

A tail biopsy of the pectoral fin was performed at 3 dpf. The protocol by Kosuta *et al.* was followed for the biopsy and lysis of the tissue[3]. The tissue was lysed using 25 µl of 50 mM NaOH and 6 µl of 500 mM Tris-HCl, pH 8.0. We used 4 µl of the DNA supernatant for a PCR analysis following the standard protocol (Supplementary figure 2B). In a following analysis the amount of DNA supernatant used was increased to 8 µl. Some larvae were sacrificed, lysed with proteinase K and genotyped using the same protocol to detect contamination issues. Pictures of the tails were taken at 7 dpf under a stereo microscope by immobilizing the larvae in methyl cellulose.

## Statistics

The comparison between Ethovision and manual scoring (Supplementary fig. 1) was analyzed for normality with the Shapiro-Wilk test. It was analyzed with a Kruskal-Wallis test for multiple comparisons since it did not meet the assumption of normality.

References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3 Kosuta, C. *et al.* High-throughput DNA Extraction and Genotyping of 3dpf Zebrafish Larvae by Fin Clipping. *J Vis Exp*, 58024, doi:10.3791/58024 (2018).

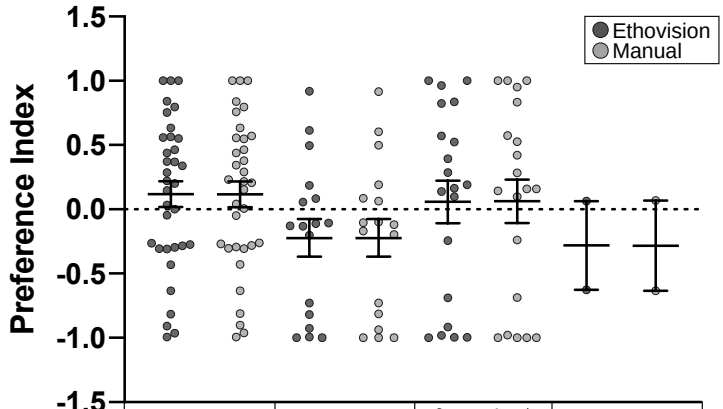

Legends

**Supplementary figure 1: Comparison between Ethovision and manual scoring.** The SPI's calculated based on the data obtained from the Ethovision software (only recordings lasting 900 s were included) were compared to the SPI's calculated based on the data obtained after manual scoring of the videos. No significant differences were detected.

**Supplementary figure 2: Genotyping techniques.** A) Pictures of larvae that underwent the tail biopsy procedure at 3 dpf and of age matched uncut controls were taken at 7dpf. A majority of larvae regenerate tails well (third row) and look very similar to uncut controls (first row), however some larvae heal abnormally and get a crooked tail (second row). B) An overview of the primers and enzymes used to genotype the larvae.

**Supplementary figure 1**

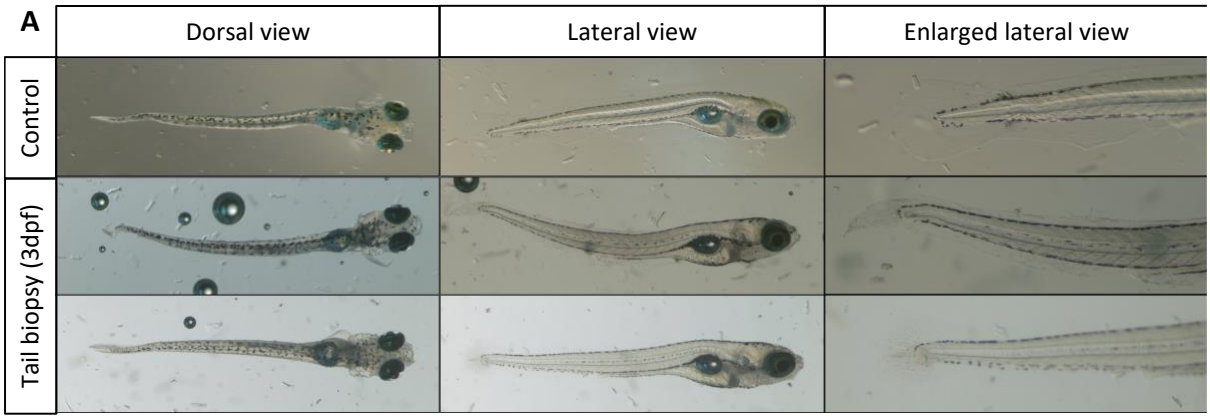
## Comparison between Ethovision and Manual scoring




| | AB | | *fam57ba*$^{+/-}$ *fam57bb*$^{+/-}$ | | *fam57ba*$^{+/-}$ *cdipt*$^{+/-}$ *fam57bb*$^{+/-}$ | | *cdipt*$^{+/-}$ | |
|---|---|---|---|---|---|---|---|---|
| n value | 34 | | 17 | | 20 | | 2 | |
| mean | 0.12 | 0.12 | -0.22 | -0.22 | 0.06 | 0.06 | -0.28 | -0.28 |
| std error | ±0.10 | ±0.10 | ±0.15 | ±0.15 | ±0.17 | ±0.17 | ±0.34 | ±0.35 |

## Supplementary figure 2

**A**




**B**

| Gene | Forward primer | Reverse primer | Enzyme | PCR product size | Cut product sizes |
|---|---|---|---|---|---|
| **fam57ba** | TTGGTTTCAAGT GTCTCCTGTC | TCGCACTCCTAC ACATCTGG | BtgZ1 | 330 | 236 + 94 |
| **fam57bb** | GAGATGAACCG ATTGTCAACAT | GTACCTAATAAA GTGGCCGGTG | Ear1 | 397 | 270 + 127 |
| **cdipt** | CCCACACGTTTA ATAGGATGTGC | TTTGTACCTAAG GACGAGGCAC | ApaL1 | 238 | 206 + 19 |