Effects of the major formaldehyde catalyzer ADH5 on phenotypes of Fanconi anemia zebrafish model

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Abstract

Background

Fanconi anemia (FA) is a devastating hereditary disorder for which we desperately need a novel therapeutic strategy. It is caused by mutations in one of at least 22 genes in the FA pathway and is characterized by developmental abnormalities, bone marrow failure, and cancer predisposition. The FA pathway is required for the efficient repair of damaged DNA, including interstrand cross-links (ICL). Recent studies indicate formaldehyde as an ultimate endogenous cause of DNA damage in FA pathophysiology. Formaldehyde can form DNA adducts as well as ICLs by inducing covalent linkages between opposite strands of double-stranded DNA.

Methods and Results

In this study, we generated a disease model of FA in zebrafish by disrupting the *ube2t* or *fancd2* gene, which resulted in a striking phenotype of female-to-male sex reversal. Since formaldehyde is detoxified from the body by alcohol dehydrogenase 5 (ADH5), we generated $fancd2^{-/-}/adh5^{-/-}$ zebrafish. We observed a body size reduction and a lower number of mature spermatozoa than wild-type or single knockout zebrafish. To evaluate if increased activity in ADH5 can affect the FA phenotype, we overexpressed human *ADH5* in $fancd2^{-/-}$ zebrafish. The progress of spermatogenesis seemed to be partially recovered due to ADH5 overexpression.

Conclusions

Our results suggest potential utility of an ADH5 enzyme activator as a therapeutic measure for the clearance of formaldehyde and treatment of FA.

Introduction

Fanconi anemia (FA) is caused by mutations in any one of at least 22 FA genes and is characterized by congenital growth abnormalities, bone marrow failure (BMF), and cancer predisposition. Defective DNA repair in FA cells can lead to higher levels of DNA damage or genomic instability such as mutations and translocations, that result in hematopoietic stem cell failure or acute myeloblastic leukemia (AML) and other blood and solid tumors, respectively[1]. FA cells show high sensitivity to DNA cross-linking agents like mitomycin (MMC) and diepoxybutane (DEB). After MMC treatment, FA cells exhibit higher levels of chromosome aberrations due to deficient repair of ICLs[2]. MMC and DEB have been widely used for FA diagnosis with high sensitivity, specificity, and reproducibility. The current standard of care for BMF in children with FA is hematopoietic stem cell transplantation (HSCT).

Previous studies have indicated that combined knockout mouse models of *adh5* or *aldh2* with *fancd2* develop catastrophic phenotypes[3,4]. ADH5 is a major formaldehyde degrading enzyme, while ALDH2 mainly catalyzes acetaldehyde. It is well known that a variant *ALDH2* allele (i.e., *ALDH2*2*, or *rs671*) encoding E504K missense mutation is prevalent among the East Asian population. This allele acts in a dominant negative manner, and an individual with heterozygous variant displays a flushing response after drinking alcohol. The variant also predisposes a habitual drinker to highly increased risk of esophageal cancer[5]. Our previous study also provided evidence that the *ALHD2* variant allele accelerated the progression of BMF in FA[6]. Furthermore, we have recently identified a novel inherited BMF syndrome, Aldehyde Degradation Deficiency Syndrome (ADDS), which is caused by combined defects in *ADH5* and *ALDH2*[7,8,9]. ADDS patients exhibited increased levels of sister chromatid exchanges (SCE) in PHA-stimulated lymphoblasts, suggesting accumulated DNA damage from endogenous formaldehyde. Both FA and ADDS model cell lines showed high sensitivity to formaldehyde[8,10]. Apart from absence of physical abnormalities in ADDS and rare occurrence of mental retardation in FA, the two disorders share similar clinical features including skin

pigmentation, microcephaly, short stature, aplastic anemia (AA), BMF, MDS or AML, and often require hematopoietic stem cell transplantation (HSCT). The FA repair pathway and the ADH5/ALDH2 aldehyde catabolism unit constitute a common pathway for suppressing formaldehyde-induced DNA damage. Treatment with the ALDH2 activator C1, modestly reversed the defective hematopoietic differentiation in ADDS-derived iPS cell models[8]. In the current study, we wanted to interrogate whether the modulation of ADH5 gene can affect the FA phenotype in a zebrafish model system, investigating the possibility that ADH5 has potential as a therapeutic target for FA.

Zebrafish seems usefull as a disease model for FA research due to their prominent phenotype of female-to-male sex reversal.[11] It has been reported that the complete female-to-male sex reversal was observed in knockouts for 12 FA genes [12]. Unlike mammals and medaka which have an XX/XY sex-determination system, zebrafish have no obvious heteromorphic sex chromosomes, and their gonads can develop into either ovaries or testes[13,14,15]. For example, *fancl*^{-/-} zebrafish, gonads become masculinized to testes because of an abnormal increase in programmed germ cell death via oocyte apoptosis, and as a result, *fancl*^{-/-} develop into males. Programmed germ cell death is mediated by p53, and loss of *p53* in *fancl* mutants rescues the sex reversal phenotype by reducing germ cell death[11].

To investigate the function of formaldehyde degrading enzymes in the absence of FA genes, we created several zebrafish models including *ube2t(fanct)*[-/-]*, fancd2*^{-/-}, and *adh5*^{-/-}. We also generated an ADDS model zebrafish. We further created *fancd2/adh5* double knockout mutants. To learn if overexpression of human ADH5 could rescue the phenotype in FA-deficient zebrafish, we used the Tol2 transposon system to generate a transgenic zebrafish by insertional mutagenesis of human ADH5 into *fancd2* single knockout zebrafish. Our study provides new insights into the relationship between endogenous formaldehyde and zebrafish sex determination. In conclusion, we suggest that artificial activation of ADH5 might alleviate the FA or ADDS phenotype.

Manuscript

Materials and Methods

Plasmid construction, sequencing, and transfection

Plasmids were prepared using the GeneElute kit (Sigma-Aldrich) according to manufacturer instructions. The pT2AL200R150G-GFP (kindly provided by Kawakami lab, National Institute of Genetics) plasmid was digested with restriction enzymes BamH1 and BsrG1, and a Gateway-Flag system was inserted using an In-Fusion kit (Clontech). Human full-length (1122 bp) *ADH5* cDNA from the hADH5 cDNA-pDONR201 entry vector (Biological Resource Center, National Institute of Technology and Evolution) was transferred to the pT2AL200R150G-Gateway-Flag destination vector using an LR clonase (Invitrogen). Zebrafish full-length *aldh2.2* (1548 bp) and *aldh2.1* (1548 bp) were isolated by PCR using the reverse transcription (RT) product derived from AB adults and cloned into pcDNA3.1-Gateway-Flag/GFP using LR reaction kit, respectively. The *aldh2.2* gene was also inserted into the pT2AL200R150G vector using a primer pair that incorporates the Flag tag. The *aldh2.2* c.1507G>A mutation was further introduced by inverse PCR and In-fusion Kit (Clonech). PrimeScript^{TM} RT reagent kit with gDNA Eraser (TaKaRa) was used for cDNA synthesis. 293T cells were cultured in DMEM (high glucose) (Nacalai Tesque) supplemented with 10% Fetal Bovine Serum (FBS) at 37°C with 5% CO_2, and transfected with plasmids using Lipofectamine 3000 kit according to manufacturer instructions.

Western blotting analysis

Zebrafish tail fin (~3 mm) was amputated, put into 200 μL 1% SDS, and boiled at 95°C for 10 min. Then tissues were sonicated and centrifuged at 14000 g for 15 min. Lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked and incubated with the indicated antibodies. The antibodies used in this study are listed in Supplementary Table 1.

Zebrafish regular maintenance and crossing

AB zebrafish were used in this study. Adult zebrafish (90 dpf) were kept in a custom-built recycling water system with approximately ~20 fish per 1L tank. Water conductivity

was adjusted to 350-450 μS and pH to 7.0-8.0. The fish were maintained at a water temperature of 28°C with alternating light (14 h) and dark (10 h) phases. Zebrafish were fed with a powdered bait or artemia twice a day. Breeding tanks with 2 males and 2 females were used for overnight breeding, separating eggs from adults. Collected eggs were transferred to a 10 cm cell culture dish with 1/3 diluted ringer solution containing methylene blue. Larvae were transferred to plastic cups with 150 mL of recycling water from 5 dpf. They were fed with 2000 rotifers (*Brachionus calyciflorus*) per day from 5 dpf to 12 dpf[17] followed by daily artemia feeding.

Microinjection of zebrafish embryos

2 males were kept separated from 2 females by a plastic mesh in a breeding tank for one night. The following morning males and females were put together in the upper chamber above the plastic mesh to allow fertilized eggs to fall into the lower chamber. Fertilized eggs were collected within 20 mins. To create a microinjection chamber, molds are placed in a petri dish and molten agarose is poured into it. Before injection, 1.5 μL solution containing 50 ng plasmid and transposase mRNA is added to the needle. Microinjection was done with the micromanipulator and the needle into the cytoplasm of embryos. Following injection, embryos are transferred to a new dish and incubated at 28°C.

Zebrafish genotyping

The fish were put in 100 ml of anesthetic solution (0.16 mg/mL Ethyl m-Aminobenzoate Methane sulfonate), and 1 mm of zebrafish tail fin was amputated to 25 μL 25 mM NaOH/ 0.2 mM EDTA in 8-strip PCR tubes. Tubes were heated at 95°C for 10 min followed by cooling to 4°C, and then 25 μL 40 mM Tris-HCl (pH 8.0) was added. The solution was mixed by vortex and spun down. PCR was carried out using KOD FX kit (TOYOBO) and the primers used in this study are listed in Supplementary Table 2. PCR products were analyzed by the heteroduplex mobility assay using MCE-202 MultiNA microchip electrophoresis system (Shimadzu, Kyoto).

H&E stained slide analysis

Zebrafish were sacrificed by freezing and dipped in Davidson's fixative solution immediately. For complete fixation, the tube was shaken gently for 72 hours. The H&E

stained slides were made by the Center for Anatomical, Pathological, and Forensic Medical Research, Kyoto University Graduate School of Medicine. Images were captured by using a BZ-9000 microscope and BZ-II image analysis application software (Keyence).

Fish sperm collection and counting.

A group of male and female fish were kept in a breeding box overnight in the same condition as single-cell embryos collecting. On the following morning, males were anesthetized in 100 ml of anesthetic solution and were wiped dry. After drying, the fish were rinsed in sperm immobilizing medium (ZSI: 140 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), buffered to pH 8.5 with 1.0 M NaOH), and fresh seminal fluid was obtained by carefully squeezing the ventral area of the fish[18]. 1 µl of seminal fluid from each fish was diluted in 200 µl ZSI, and observed with the hemocytometer and a microscope.

Statistical analysis

P-values were calculated using Prism software (Graphpad, USA).

Results

Generation of FA zebrafish models

The FA knockout zebrafish models were made by microinjection to wild-type fish with the CRISPR/Cas9 plasmid and sgRNAs targeting *ube2t* or *fancd2* (**Figure 1A** and **B**). We outbred the fish with germline transmitting mutations to generate first filial generation fish (F1) fish heterozygous for frameshift mutations. These fish were genotyped in adulthood. Details of the mutant alleles including the size of insertion (*ube2t* +5) or deletion (*fancd2* △8) are shown in **Figure 1A** and **B**. By submitting zebrafish genome DNA PCR products to MultiNA analysis, we could detect a band shift in homozygous mutant fish compared to wild-type fish. If the wild-type and mutant PCR products have different lengths, their hybrids from heterozygous fish will have a different spatial structure, contributing to a higher position in the microchips, resulting in 3 or 4 bands being present in the MultiNA analysis of heterozygous fish (the heteroduplex migration

assay, **Figure 1A** and **B**). Homozygous knockout fish were observed among the surviving adults for both of the genes, indicating that there is no lethality at early developmental stages. Next, we crossed *ube2t*^{-/-} males and *ube2t*^{+/-} females, or *fancd2*^{-/-} males and *fancd2*^{+/-} females. The distribution of the genotype was at the expected Mendelian ratios for both of these mutant fish (**Figure 1C** and **D**). Similar to previous studies, our FA gene knockout fish exhibited a female-to-male sex reversal phenotype (**Figure 1C** and **D**)[11,12].

Generation of ADDS zebrafish models

Next, we aimed to make an ADDS zebrafish model, which may provide a novel insight into how aldehydes affect physiological features of zebrafish. First, we generated *adh5*^{-/-} fish in a similar way as described above. Details of the position of the sgRNA targeting sequence and the mutant alleles (*adh5*^{-/-} -2/+10) are described in **Figure 2A**. There were no differences in phenotype between wild-type and *adh5*^{-/-} fish, and the sex ratio of *adh5*^{-/-} fish was not altered (**Figure 2B**). Next, we wished to make ALDH2 deficient zebrafish model. To mimic the human ALDH2 rs671 variant, we chose the transgenic expression of an *aldh2* variant instead of pursuing an entire knockout of *aldh2* isoforms. We first compared the cDNA sequence between human *ALDH2* and zebrafish *aldh2.1* and *aldh2.2*[16]. The high degree of homology between them supported the idea that expression of the fish ALDH2-E503K may exhibit a similar dominant negative effect as the human ALDH2-E504K (**Figure 2C**). To ensure the expression of the variant isoform ALDH2.2 can suppress ALDH2.1 activity, we confirmed that ALDH2.1 and ALDH2.2 can physically associate with each other by Co-immunoprecipitation (Co-IP) (**Figure 2D**).

By microinjection of the Tol2 transposon system consisting of *aldh2.2* c.1507G>A cDNA to zebrafish eggs obtained from *adh5*^{-/-} crossing, we succeeded in obtaining the ADDS model fish that integrated the *aldh2.2* c.1507G>A transgene (**Figure2E**). The genomic PCR and western blotting analysis showed that *aldh2.2* c.1507G>A could be inherited to the next generation (**Figure 2F**). We crossed the ADDS model and *adh5*^{+/-} fish, and noticed that the body size of ADDS model fish was smaller than *adh5*^{+/-}/*aldh2.2* c.1507G>A fish at 60 dpf (**Figure 2G**). Even though the appearance

of the adult fish were similar between these mutant fishes (**Figure 2H**), our data suggested a slightly slower growth rate in the ADDS model fish. The sex ratio was also normal, which was different from the FA model fish (**Figure 2I**). In contrast to the mouse ADDS model[7], our fish ADDS model appeared to be quite healthy.

The *fancd2-/-/adh5-/-* zebrafish displayed a body size reduction

By crossing the *fancd2-/-* males and *adh5-/-* females, *fancd2* and *adh5* heterozygous individuals (*fancd2+/-/adh5+/-*) were obtained (**Figure 3A**). After sexual maturity (90 days post-fertilization, 90 dpf), *fancd2+/-/adh5+/-* males and females (P1: the first parent generation) were crossed. The F1 fishes were checked for genotype by MultiNA analysis. If the *fancd2/adh5* double mutant homozygous fish (*fancd2-/-/adh5-/-*) could survive to adulthood, they would be 6.25% (1/16) of all the fish according to Mendelian inheritance. 14 fish (4.08%) among 343 F1 fish were found to be *fancd2-/-/adh5-/-* (**Figure 3B**). These results indicate the genotype distribution conforms to Mendelian inheritance (chi-square p-value=0.0824). These *fancd2-/-/adh5-/-* fish were all fertile males and survived to adulthood (**Figure 3B**). In contrast to the *fancd2-/-/adh5-/-* mice, which showed a severe phenotype, the double mutation of *fancd2* and *adh5* in zebrafish did not affect viability. Furthermore, we found that there was no significant difference in gross appearance between fish with the different genotypes, but we noticed that *fancd2-/-/adh5-/-* fish are smaller than the other genotypes at 60 dpf (**Figure 3C**). We then repeated backcrossing between *fancd2-/-/adh5-/-* male fish and *fancd2+/-/adh5-/-* female fish and obtained 19 fish (F2). Genotyping showed that 10 (52.63%) of them were *fancd2-/-/adh5-/-* fish (**Figure 3D**). The *fancd2-/-/adh5-/-* fish also showed a shorter body length even after they grow to adulthood, at 90 dpf (**Figure 3E**).

The *fancd2-/-/adh5-/-* zebrafish showed a lower number of mature spermatozoa

To investigate the histological changes, we made hematoxylin-eosin (H&E)-stained specimens of fish with the four genotypes (wild-type, *fancd2-/-*, *adh5-/-*, *fancd2-/-/adh5-/-*) with assistance from the Center for Anatomical, Pathological and Forensic Medical

Research, Kyoto University Graduate School of Medicine. The morphology of the kidney, heart and intestine appeared normal in *fancd2*^{-/-}/*adh5*^{-/-} fish, compared to those of the other three genotypes (Supplementary Figure 1). However, we found that the number of mature spermatozoa was reduced in *fancd2*^{-/-} and *fancd2*^{-/-}/*adh5*^{-/-} fish compared to wild-type and *adh5*^{-/-} fish (**Figure 4A**). Previous studies in zebrafish have shown that defects in FA genes such as *FANCD1, FANCJ, and RAD51*, result in loss of fertility. However, we found that the *fancd2*^{-/-} fish were fertile[12,19]. Further, we collected sperm of wild-type, *fancd2*^{-/-}, *adh5*^{-/-}, *fancd2*^{-/-}/*adh5*^{-/-} fish and counted the sperm numbers. As expected, *fancd2*^{-/-} fish have a highly reduced number of mature spermatozoa compared to wild-type fish. Although the number of mature spermatozoa appeared to be unaffected by single knockout of *adh5*, the number in *fancd2*^{-/-}/*adh5*^{-/-} double knockout fish was even lower than those in *fancd2*^{-/-} fish (**Figure 4B**). These results reflect an important role of FANCD2 in meiotic homologous recombination, and also indicate that ADH5 contributes to the progression of spermatogenesis.

Human *ADH5* overexpression partially recovered spermatogenesis in *fancd2*^{-/-} zebrafish

To date, few studies have investigated the effects of overexpression of *ADH5* (h*ADH5*-O/E) on FA models. We introduced human *ADH5* in the *fancd2*^{-/-} zebrafish model (*fancd2*^{-/-}/h*ADH5*-O/E) to examine whether the human ADH5 could rescue the phenotype of *fancd2* deficiency. *fancd2*^{-/-}/h*ADH5*-O/E zebrafish were made by microinjection of the Tol2 transposon system consisting of h*ADH5* cDNA to zebrafish eggs from crossing *fancd2*^{-/-} with *fancd2*^{+/-} (**Figure 5A**). We succeeded to isolate two fish integrating h*ADH5* cDNA, and confirmed that the h*ADH5* sequence was inherited to the next generation (**Figure 5B**). Additionally, by western blotting analysis, ADH5 protein with a Flag-tag was detected in the next generation, confirming the expression of the inserted h*ADH5* gene. In 100 zebrafish with hADH5 overexpression, all of the *fancd2*^{-/-} fishes are male (n=49), while in *fancd2*^{+/-}, half of them are females. Thus, no *fancd2*^{-/-} female was born, and hADH5 overexpression could not rescue the sexual reversal in *fancd2*^{+/-} zebrafish (**Figure 5C**). However, given the loss of ADH5 aggravated the reduction of mature

spermatozoa number in *fancd2*^{-/-} fish, we wondered if overexpression of human ADH5 could rescue this phenotype. We counted the mature spermatozoa from the fish of three genotypes *in vitro*. In *fancd2*^{-/-}/h*ADH5*-O/E fish, the mature spermatozoa number was significantly increased (**Figure 5D**). Thus, we concluded that the overexpression of human *ADH5* could partially rescue the phenotype of the FA zebrafish model, and supported the idea that an activator of ADH5 might provide a benefit for patients with FA.

Discussion

The purpose of the current study was to observe the effects of *adh5*, the gene coding a formaldehyde degrading enzyme, in zebrafish. To this aim, we developed several disease models in zebrafish for FA as well as an FA-related disorder ADDS, and deleted or overexpressed *ADH5* in the FA model. Previous studies have found that mouse models, including *adh5*^{-/-}/*fancd2*^{-/-}, *aldh2*^{-/-}/*fancd2*^{-/-} and *adh5*^{-/-}/*aldh2*^{-/-}, die at an early age[3,4,7]. In contrast, all the generated zebrafish models survived to adulthood, indicating that the combined deletions of *fancd2* and *adh5* did not overtly compromise viability in zebrafish. Our ADDS model zebrafish developed very few phenotypes and we found only mild body size reduction. We also generated *fancd2*^{-/-}/*adh5*^{-/-}/*aldh2.2* c.1505G>A or *fancd2*^{-/-}/*aldh2.2* c.1505G>A zebrafish (data not shown), and the phenotype was not intensified in *fancd2*^{-/-} or *fancd2*^{-/-}/*adh5*^{-/-} by the dominant negative ALDH2 (E503K) expression. It is still possible that the complete deletion of both *aldh2.1* and *aldh2.2* might display more robust effects on the phenotype. A recently published study indicated that *aldh2*^{-/-} (and *adh5*^{-/-}) mutant embryos of zebrafish displayed a higher sensitivity to formaldehyde, consistent with this possibility[20].

  We obtained *fancd2*^{-/-} zebrafish that were all male, and their number of sperm was drastically reduced. However, other than these, *fancd2*^{-/-} zebrafish displayed no obvious phenotypes that were similar to human FA. For adult *fancd2*^{-/-}/*adh5*^{-/-} zebrafish, we noticed a reduction in body size, compared to wild-type and *fancd2*^{-/-} fish. Thus, the somatic development was mildly compromised, probably because more replication stress

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or cell death occurs in the development process due to the inability to catalyze endogenously generated formaldehyde. It is also possible that the process similar to the proposed endocrine changes caused by accumulated DNA damage may account for the decrease in appetite and reduction in body size[21].

Though the progress of spermatogenesis is compromised in *fancd2*^{-/-} fish, the available sperm was enough for the fertilization of eggs. Hence, the defective repair of ICL affects spermatogenesis more crucially than that in somatic development. Moreover, the loss of *adh5* aggravates the deficiency in the progress of spermatogenesis. We also created another *fancd2*^{-/-} zebrafish disease model with human *ADH5* overexpression. No *fancd2*^{-/-} females were born, however, the decreased number of spermatozoa was mildly rescued. These results suggest that formaldehyde might be generated during the process of spermatogenesis and its catabolism plays a role in the production of spermatozoa. However, it is unclear how formaldehyde is generated in this process and this issue needs further exploration.

In conclusion, our experiments confirmed that zebrafish with combined loss of *adh5* and *fancd2* could survive to adulthood and be used as a disease model for the research on the function of *adh5* in a genome instability background. We have conducted a basic analysis and learned that *adh5* contributes to both somatic development and spermatogenesis. In addition, using our *fancd2*^{-/-} zebrafish with *ADH5* overexpression, we provide insight into how *ADH5* enzyme activators could be used for the treatment of FA. The major limitation of this study is that the concentration of endogenous formaldehyde was not determined due to low sensitivity of current technologies accessible for us. Nonetheless, these results may suggest that activation of *adh5* could be a method for alleviating FA phenotypes.

Conflict of Interest

The authors declare no competing interests.

Author Contributions

M.T. and S.M. planned the study; A.M. carried out most of the experiments and analyzed data with help from Z.C., D.H., and H.H.; M.T. and A.M. wrote the paper.

Acknowledgments

Supplementary Material

The Supplementary Material for this article can be found online.

Data Availability Statement

All relevant data are available from the authors upon reasonable request. All the genotypes of zebrafish generated in this study were disposed to the National BioResource Project, Zebrafish, Core Institution: NZC (https://shigen.nig.ac.jp/zebra/index.html), Japan.

Compliance with Ethical Standards:

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The animal experiment committee of Graduate School of Biostudies, Kyoto University has approved this study.

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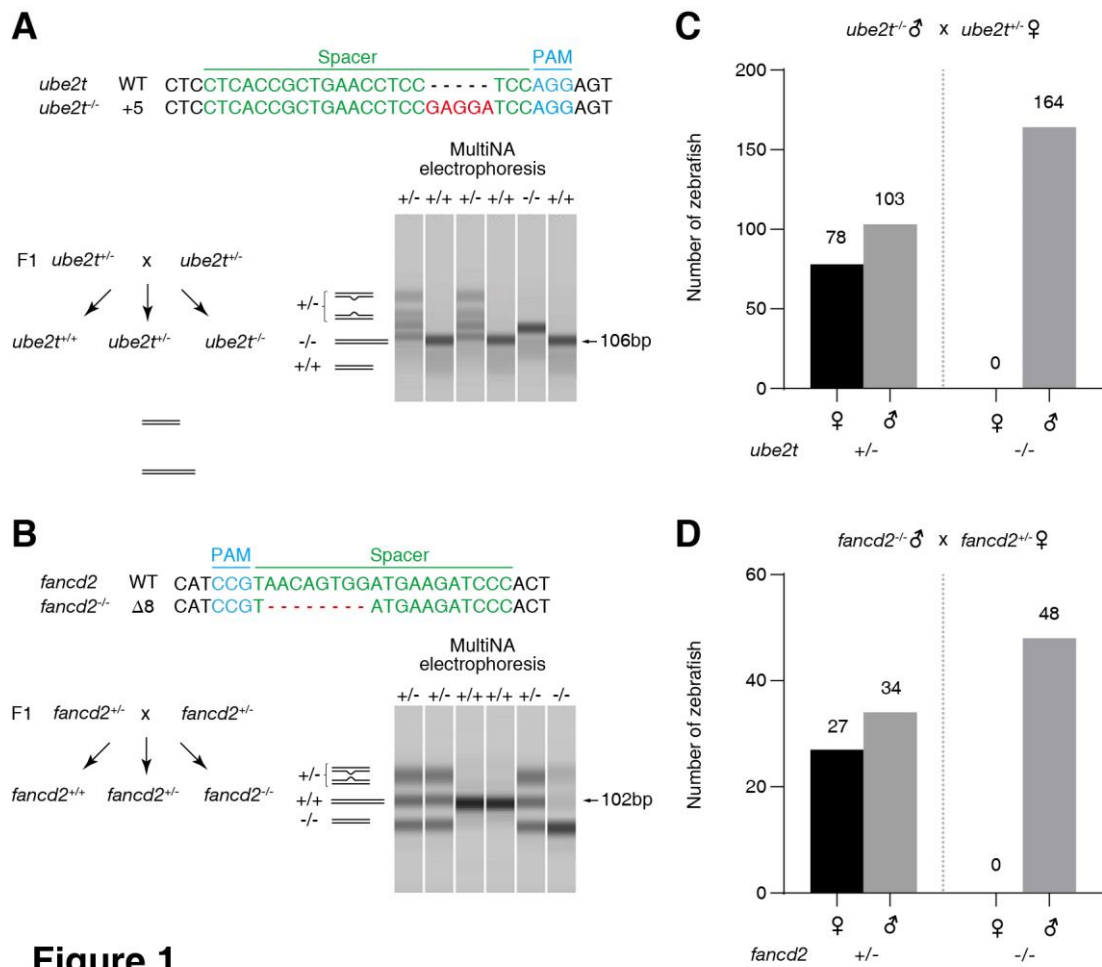
Figure 1

Figure 1. Generation of FA model *ube2t* or *fancd2* single knockout zebrafish. sgRNA targeting position and frameshift pattern of (**A**) *ube2t$^{-/-}$* and (**B**) *fancd2$^{-/-}$* zebrafish. The genomic PCR products were subjected to the heteroduplex mobility assay (HMA) using MultiNA. The HMA can discriminate genotypes by different electrophoresis patterns. The sex ratio of (**C**)*ube2t$^{-/-}$* and (**D**) *fancd2$^{-/-}$* homozygous mutants. The absence of females in *ube2t$^{-/-}$* or *fancd2$^{-/-}$* homozygous mutants is due to sex reversal.
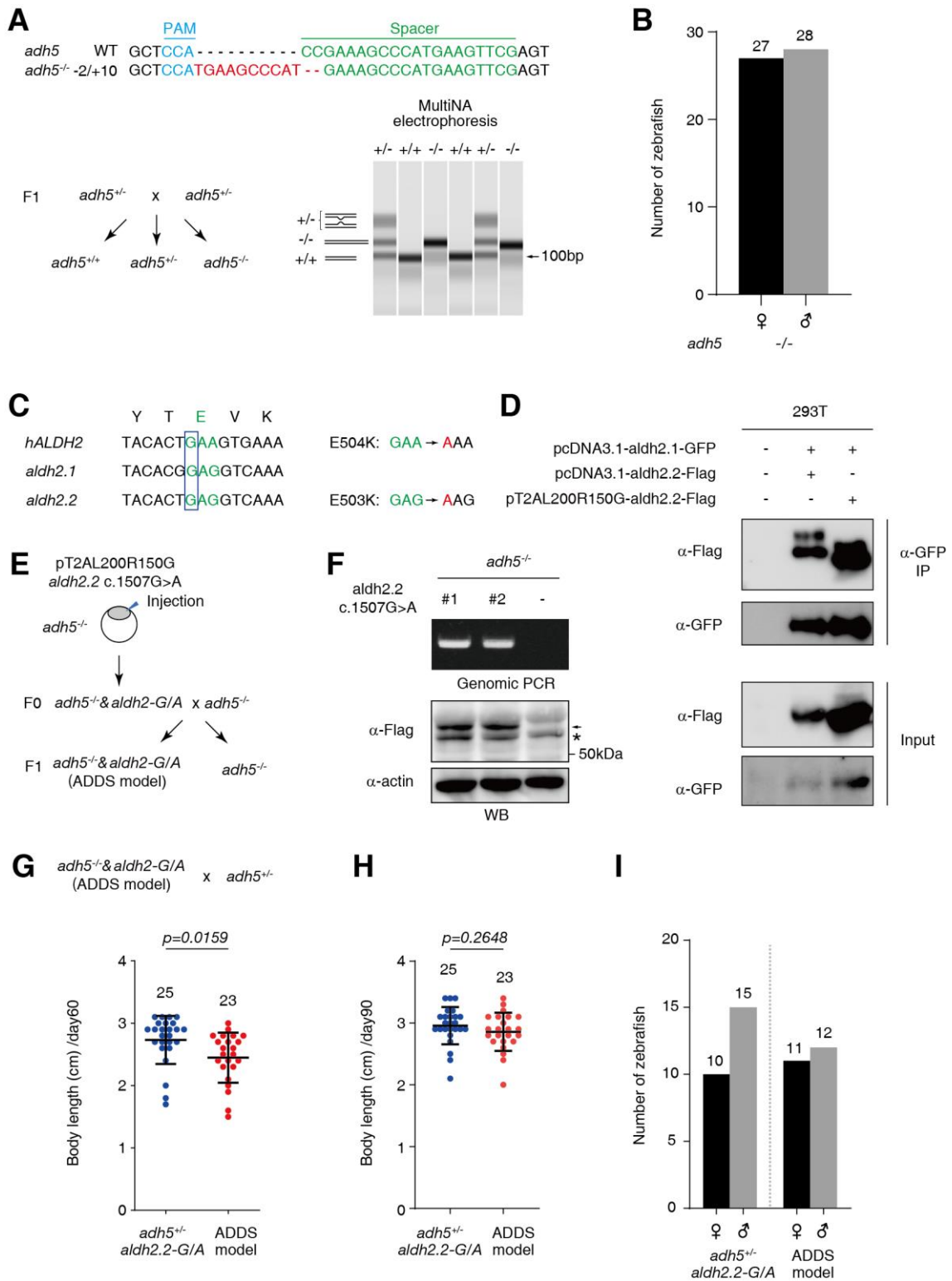
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Figure 2

Figure 2. Generation of ADDS model zebrafish. (**A**) sgRNA targeting position and frameshift pattern of *adh5⁻ᐟ⁻*. MultiNA analysis images showed 3 bands representing the indicated genotype of zebrafish. (**B**) The sex ratio of *adh5⁻ᐟ⁻* homozygous mutants. (**C**) Alignments of part of the human *ALDH2* and zebrafish *aldh2.1*, *aldh2.2*. The green fonts represent the codon of ALDH2 encoding E504 (human) or E503 (zebrafish). The E504K missense mutation in human exhibits dominant negative effects in *ALDH2*2*. (**D**) Co-immunoprecipitation (Co-IP) analysis between zebrafish ALDH2.1 and ALDH2.2. After 293T cells were transfected with indicated plasmids, the cell lysed were subjected to Co-IP by anti-GFP, and precipitated proteins were detected by western blotting with anti-Flag. The size difference between aldh2.2 cloned in pcDNA3.1 and in pT2AL200R150G was caused by the different length of linker between aldh2.2 and Flag tag. (**E**) Scheme of generating ADDS zebrafish. *adh5⁻ᐟ⁻* fish were injected with pT2AL200R150G *aldh2.2* c.1507G>A and Tol2 transposon mRNA. F0: the injected filial generation. F1: the first filial generation. (**F**) Genomic PCR and western blotting (WB) analysis of the *aldh2.2* c.1507G>A positive fish. * Nonspecific band. Body length comparison between ADDS and *adh5⁺ᐟ⁻ aldh2.2* c.1507G>A fish at (**G**) 60 dpf and (**H**) 90 dpf. The P values were calculated using a two-sided Student's t-test.
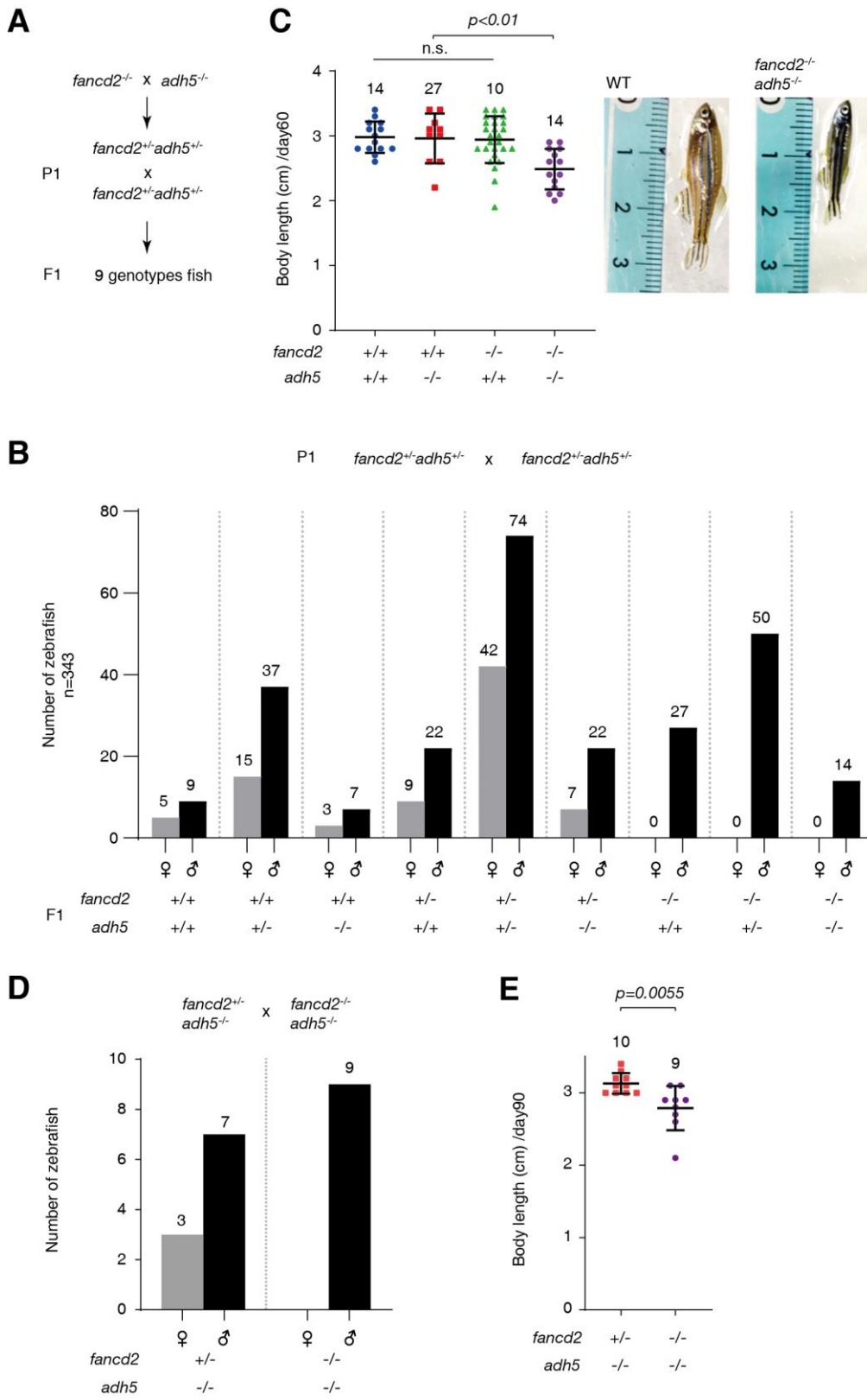
Figure 3

Figure 3. Generation of *fancd2 adh5* double knockout zebrafish. (**A**) Scheme of generating *fancd2^{-/-}/adh5^{-/-}* fish. (**B**) Genotype and sex ratios of F1 fish crossed by P1 fish (*fancd2^{+/-}/adh5^{+/-}*). The ratio of genotypes has no significant difference from the expected ratio according to Mendelian inheritance. (**C**) Body length comparison between *fancd2^{-/-}/adh5^{-/-}* zebrafish with-wild type or single knockout zebrafish. 60dpf male fish was used for body length measure. Representative images are shown in the right panel. The P values were calculated using 1-way ANOVA with Tukey's multiple-comparisons test. (**D**) The sex ratio of indicated genotype fish. (**E**) Body length comparison between *fancd2^{-/-}/adh5^{-/-}* and *fancd2^{+/-}/adh5^{-/-}*. 90dpf male fish was used for body length measure. The P values were calculated using a two-sided Student's t-test.
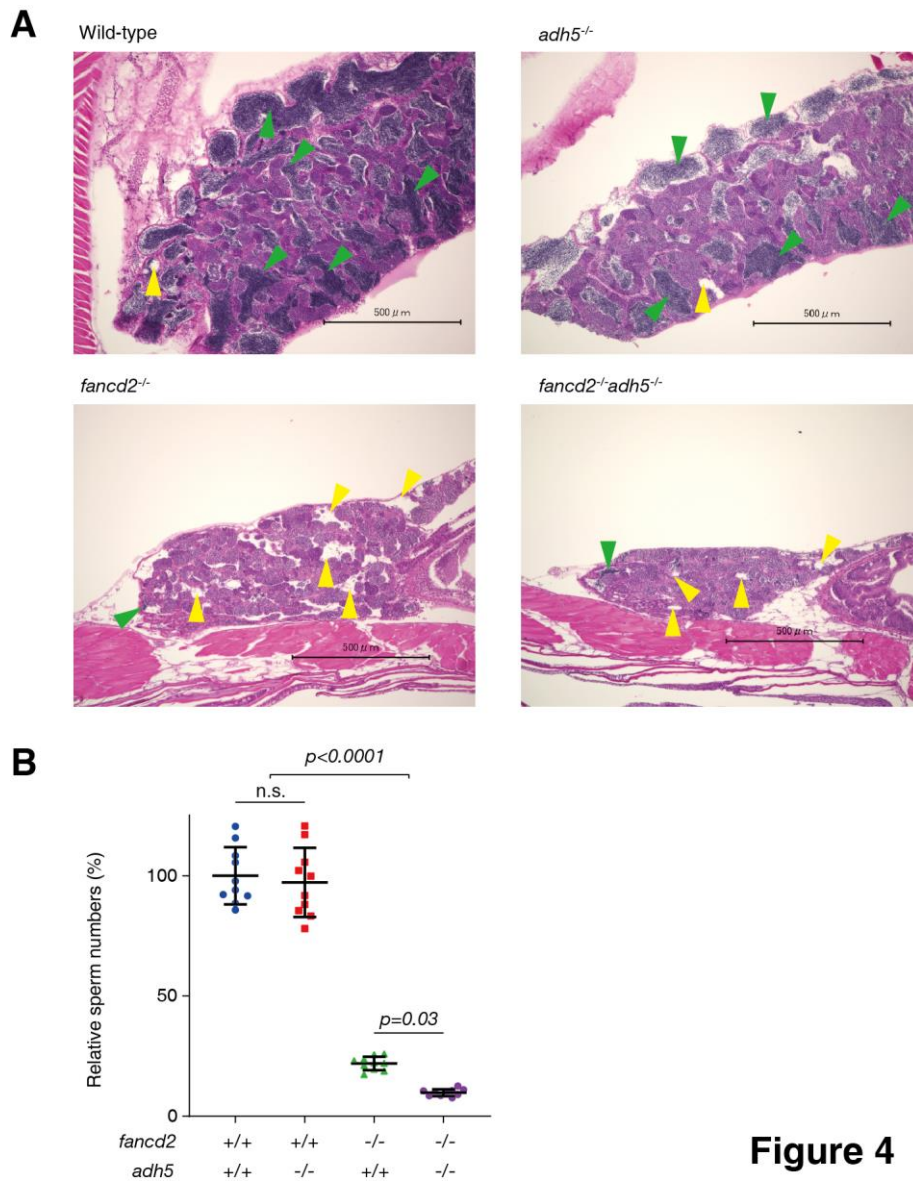
Figure 4. ADH5 contributes to the progress of spermatogenesis in a model zebrafish of FA. (**A**) Histology of testis in wild-type(WT), *fancd2*^{-/-}, *adh5*^{-/-}, *fancd2*^{-/-}/*adh5*^{-/-} male zebrafish. Adult male fish were used for slide making, H&E stained. Yellow triangle mark: empty seminiferous tubules. Green triangle mark: mature spermatozoa. Scale bar: 500 μm. (**B**) Relative sperm numbers of wild-type, *fancd2*^{-/-}, *adh5*^{-/-}, *fancd2*^{-/-}/*adh5*^{-/-} male zebrafish. Male fish after 90dpf were used for sperm number counting. The P values were calculated using 1-way ANOVA with Tukey's multiple-comparisons test.
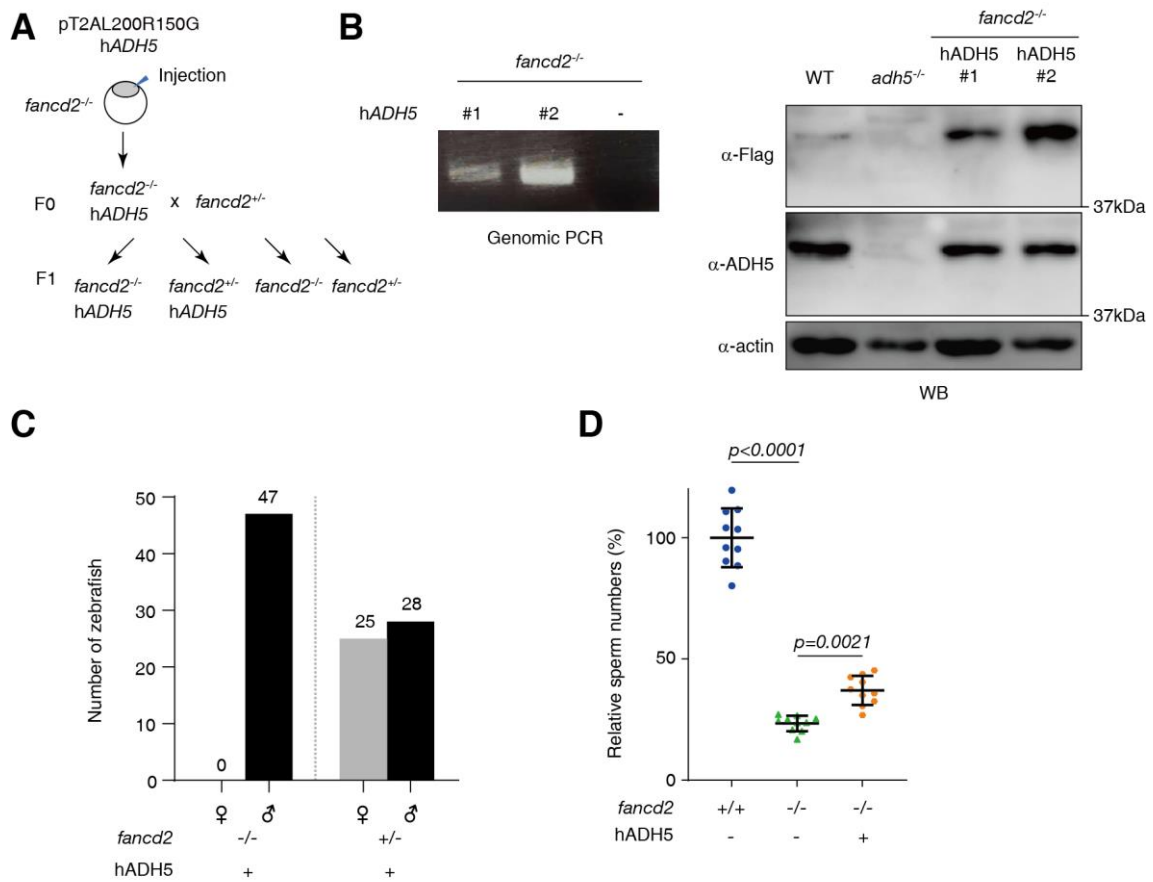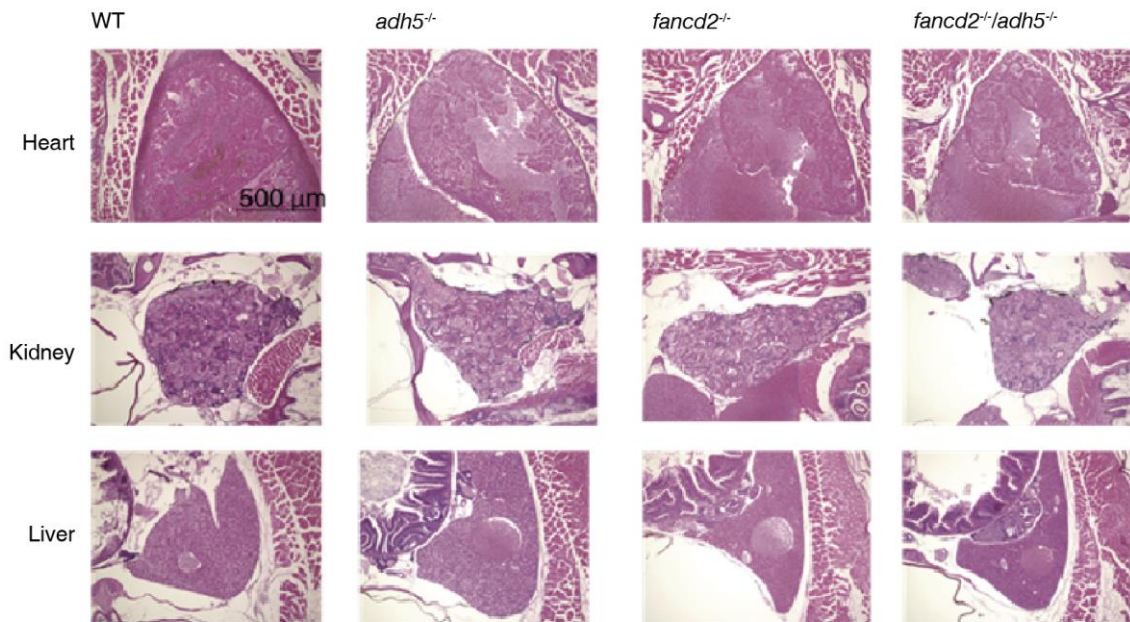
Figure 5

Figure 5. Spermatogenesis in _fancd2-/-_ fish was partially recovered due to the overexpression of human ADH5. (**A**) Scheme of generating hADH5 overexpression zebrafish. The _fancd2-/-_ fish were injected with pT2AL200R150G-hADH5-Flag and Tol2 transposon mRNA. (**B**) Genomic PCR and western blotting (WB) analysis of the hADH5 positive fish. Zebrafish ADH5 can be detected by rabbit polyclonal anti-ADH5 (Proteintech, Cat. 11051-1-AP). The absence of the band corresponding to ADH5 is observed in _adh5-/-_ zebrafish. (**C**) Sex ratios of hADH5 overexpression F2 fish. (**D**) Relative sperm numbers of wild-type, _fancd2-/-_, _fancd2-/-_/h_ADH5_-O/E male zebrafish. Male fish after 90dpf were used for sperm number counting. The P values were calculated using 1-way ANOVA with Tukey's multiple-comparisons test.

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Supplementary Material

Supplementary Figure 1

Supplementary Figure 1. Histology of heart, kidney, and livers in wile-type(WT), *fancd2*^{-/-}, *adh5*^{-/-}, *fancd2*^{-/-} *adh5*^{-/-} male zebrafish. 90dpf male fish were used for slide making. H&E stained. Scale bar: 500 μm.

Supplementary Table 1. Antibodies used in this study.

Antibodies	Source	Catalog No.	RRID
Mouse monoclonal anti-DDDDK-tag (anti-Flag)	MBL	M185-3L	AB_11123930
Rabbit polyclonal anti-ADH5	Proteintech	11051-1-AP	AB_593422
Mouse monoclonal anti-GFP	MBL	M048-3	AB_591823
Rabbit polyclonal anti-β-actin	Sigma-Aldrich	A5060	AB_476738

Supplementary Table 2. Primer sequences used in this study.

Gene	Method	Primer	
ube2t	MultiNA	KD15-407	ATGCAGAGAGTCAGTCGTCTGAAGCGGGAGATGCA
		KD15-408	CTTGTAGTTCATCCAGGCGTCCCTCTGACTGCCAG
	Sequence	KD22-12	GATGAATCTGGATGCACCTCCGGCGCACAA
		KD22-12	CCAATGTGAACACACCACCTTCATACGG
fancd2	MultiNA	KD16-1	GCATTTTCATCTGATCTAAC
		KD16-2	GTGTGTACTTACTAGACACG
	Sequence	KD16-541	AACACAGTATTTACACGACAAACGTTAAAG
		KD16-4	CAACCTTTAGTGTATACACA
adh5	MultiNA	KD17-6	CTGGTAAGCCTCTTTCCATTGAGGAGGTGG
		KD16-7	CGAGAGGTACAATGTTCAGATTAATAGTTT
	Sequence	KD17-186	GTATTAGAGCACTGGCTTACATAACCTTCG
		KD17-187	CGCCTACATCTTAGATGGGGTGAAGTTGAG
Zebrafish *aldh2.2* cDNA	In-Fusion	KD19-95	ATGCTGCGCACAGTTTTATCCAGAGCTTTC
		KD19-96	ATCATCTTTATAATCGGAGTTCTTCTCAGGA ACTTTAATG
aldh2.2 c.1507G>A	In-Fusion	KD19-93	AACTACACTAAGGTCAAAACGGTGACCATT
		KD19-94	GACCTTAGTGTAGTTCTCCAGTCCATACTC
Human ADH5	LR Reaction	KD16-155	CACCATGGCGAACGAGGTTATCAAGTGCA
		KD16-156	TTAAATCTTTACAACAGTTCGAATG