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Analysis of mutants from a genetic screening reveals the control of intestine and liver development by many common genes in zebrafish

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ABSTRACT

Both the intestine and liver develop from the endoderm, yet little is known how these two digestive organs share and differ in their developmental programs, at the molecular level. A classical forward genetic screen, with no gene bias, is an effective way to address this question by examining the defects of the intestine and liver in obtained mutants to assess mutated genes responsible for the development of either organ or both. We report here such a screen in zebrafish. ENU was used as the mutagen because of its high mutagenic efficiency and no site preference. Embryos were collected at 3.5dpf for RNA whole mount *in situ* hybridization with a cocktail probe of the intestine marker *ifabp* and the liver marker *lfabp* to check phenotypes and determine their parental heterozygosis. A total of 52 F2 putative mutants were identified, and those with general developmental defects were aborted. To rule out non-inheritable phenotypes caused by high mutation background, F2 putative mutants were outcrossed with wild type fish and a re-screen in F3 generations was performed. After complementation tests between F3 mutants with similar phenotypes originating from the same F2 families, a total of 37 F3 mutant lines originated from 22 F2 families were identified after screening 78 mutagenized genomes. Classification of mutant phenotypes indicated that 31 out of the 37 mutants showed defects in both the intestine and liver. In addition, four “intestine specific mutants” and two “liver specific mutants” showed selectively more severe phenotype in the intestine and liver respectively. These results suggested that the intestine and liver share a substantial number of essential genes during both organs development in zebrafish. Further studies of the mutants are likely to shed more insights into the molecular basis of the digestive system development in the zebrafish and vertebrate.

1. Introduction

Both the intestine and liver are derived from the endoderm, playing fundamental roles in vertebrate physiology [1]. Much efforts have been paid to uncover their developmental mechanisms in animal models. The TGFβ/Nodal signaling pathway is at the top of the molecular hierarchy and essential for endodermal cell fate determination [2]. The Zebrafish Cdx1b, a functional equivalent of mammalian Cdx2, regulates the expression of downstream factors of Nodal signaling during early endoderm formation [3,4]. The pan-endoderm factors such as members of the Gata family are essential for the intestine and liver organogenesis [5–7], while Foxa factors are required for the initiation of liver development [8]. It has been reported that the mesodermal signals FGF, WNT, BMP and RA pathways play very important roles in the anterior-posterior patterning of the gut [9–13] and the initiation and differentiation of hepatocyte [14–16]. Given that both the intestine and liver are derived from the endoderm, it is of no surprise that endodermal determination factors and mesodermal induction signals are essential for the development of both organs. On the other hand, some specific regulatory mechanisms were also reported. TOR signaling controls epithelial morphogenesis in the vertebrate intestine [17]. The Delta-Notch signaling needs to be blocked for the intestinal epithelial cells to differentiate along a secretory pathway [18], and the repression of secretory cell fate by Notch signaling is mediated by the inhibition of *ascl1a* expression [19]. Seiler discovered that smooth muscle tension induces invasive

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remodeling of the zebrafish intestine [20]. Some factors have been identified to affect liver budding and growth. For instance, Hex is crucial for the initiation and budding of the liver primordium [21], while Prox1 controls hepatocyte migration from the endodermal epithelium into the septum transversum [22].

Despite of the numerous reports on genes function in the regulation of intestine or liver development, there is no comprehensive comparison of the similarities and differences of the developmental molecular mechanisms between these two close yet distinct organs. A forward genetic screen randomly surveying the whole genome without gene predisposition may offer us an answer by studying organogenesis of the intestine and liver via genetic mutants.

The zebrafish, *Danio rerio*, has proven to be a powerful model system to study vertebrate organogenesis due to its unique characteristics and conserved molecular mechanisms [23,24]. Zebrafish are easy to raise and reach sexual maturity at around three months. Each female lays several hundred of eggs per week. The embryos are small, transparent and develop outside of the mother body, which are ideal for large-scale genetic screen [25,26]. A lot of mutagenesis screens have been conducted in the organism since the first two large-scale screens in 1996 [27,28], including efforts in searching for either the intestine [29] or liver mutants [14,30]. However, there is no report that examines both digestive organs at the same time.

While gene knock-out technologies, such as TALEN and CRISPR/Cas9, are extremely useful in mutating a target gene [31–33], forward genetic screen, though time-consuming, is still the best approach to get specific genetic mutants to study the molecular mechanisms underlying organogenesis unbiasedly. The most effective way of inducing small, intragenic lesions on a genome-wide scale is chemical mutagenesis. For zebrafish, ENU (N-ethyl-N-nitrosourea) is so far the best mutagen to introduce random point mutation into the genome [34]. The mutagenesis efficiency of the viral vector is merely one-ninth of that observed of ENU [35]. After ENU mutagenesis, F2 families can be used to screen for heterozygous mutants carrying recessive mutations based on the phenotypes revealed in F3 progenies, following Mendel's law of segregation, mutants are then proceeded to map-based cloning to identify the genes responsible for specific phenotypes.

We describe here a forward genetic screen for mutants with intestine and/or liver defects, and the mutants were classified into three categories based on their phenotypes. Further interrogations on these genetic mutants may help us better understand the development of these organs and the underlying molecular mechanisms.

2. Materials and methods

2.1. Zebrafish strains and maintenance

Zebrafish strain $AB^{tü}$ was utilized as the wild type line for outcrossing of the mutants in the study. Fishes were raised and maintained according to standard procedures [36].

2.2. Collection of fertilized eggs

Male and female fishes were separated in the crossing tank at the night before the crossing day. On the next day, the separator was removed to allow mating of the two fishes to obtain synchronized fish embryos. Fertilized eggs were collected and raised in 0.03% sea salt water at 28.5 °C. The developmental stages of the embryos were determined according to previous description [37]. Embryos for RNA whole mount *in situ* hybridization (WISH) were raised with 0.03% 1-phenyl-2-thiourea (PTU) at 12 hours post fertilization (hpf) to inhibit pigment formation, and harvested at 3.5 days post fertilization (dpf) when both the intestine and liver are morphological developed in a large part [23,38].

2.3. ENU mutagenesis and breeding of F2 families

Male fishes (AB strain) were treated with standard ENU mutagenesis procedure [39]. Survivors were outcrossed with wild type female fishes to generate F1 families. F2 families were generated by self-crossing fishes from different F1 families.

2.4. Screening procedure and classification of the mutants

Siblings from the same F2 family were randomly crossed to get F3 progeny. Around 40 embryos from each crossing pair were collected at 3.5dpf to perform WISH. The F2 parents are potential candidates for heterozygous mutants if approximately 25% of the F3 embryos show similar defects according to Mendel's law of heredity. Putative F2 mutants were outcrossed with wild type fishes to generate F3 families.

Similarly, the F4 embryos were utilized to determine F3 heterozygous mutants. Complementation tests were performed if several mutant pairs from the same F2 family exhibit similar phenotypes, and they would be assigned into the same complementation group upon positive results. Subsequently, mutants were classified into different categories based on their phenotypic characteristics (Fig. 1).

2.5. Whole-mount in situ hybridization (WISH)

Probe synthesis and whole-mount RNA *in situ* hybridization were carried out as previously described [30]. The 3.5dpf embryos were digested with Proteinase K (1:1,000, Thermo Fisher) at 37 °C for 26 min. Antisense RNA probes were labeled with digoxigenin (DIG, Roche Diagnostics). Images were taken under a Zeiss Stereo & Zoom Microscope.

3. Results

3.1. ENU mutagenesis and generation of F2 screening families

Thirty healthy male zebrafishes (AB strain) around six months old were used for ENU mutagenesis. Nine fishes survived the treatment, which were outcrossed with wild type female fishes to generate F1 families. Each founder yielded around 100 F1 offspring to grow into adulthood, generating a total of 958 F1 fishes. Fishes from different F1 families were crossed with each other rather than with wild type fishes to generate F2 families, greatly improving the screening efficiency. As a part of a large-scale screen, our screen was performed in some F2 families.

3.2. Screening in F2 families and outcrossing of the mutants

Inbreeding is the method used to identify heterozygous mutant parents. F3 embryos for screening were obtained by randomly crossing the siblings within the same F2 family. A total of 203 crossing pairs were screened, which came from 42 F2 families, among which 66 candidate mutant pairs were identified. Twenty nine pairs with ambiguous results were performed for second identification where 14 pairs were found to be false positives and excluded. Nine pairs were discarded due to their general developmental defects which would bring about the intestine and liver phenotypes as a secondary consequence. Forty three putative mutant pairs originated from 27 F2 families were chosen to

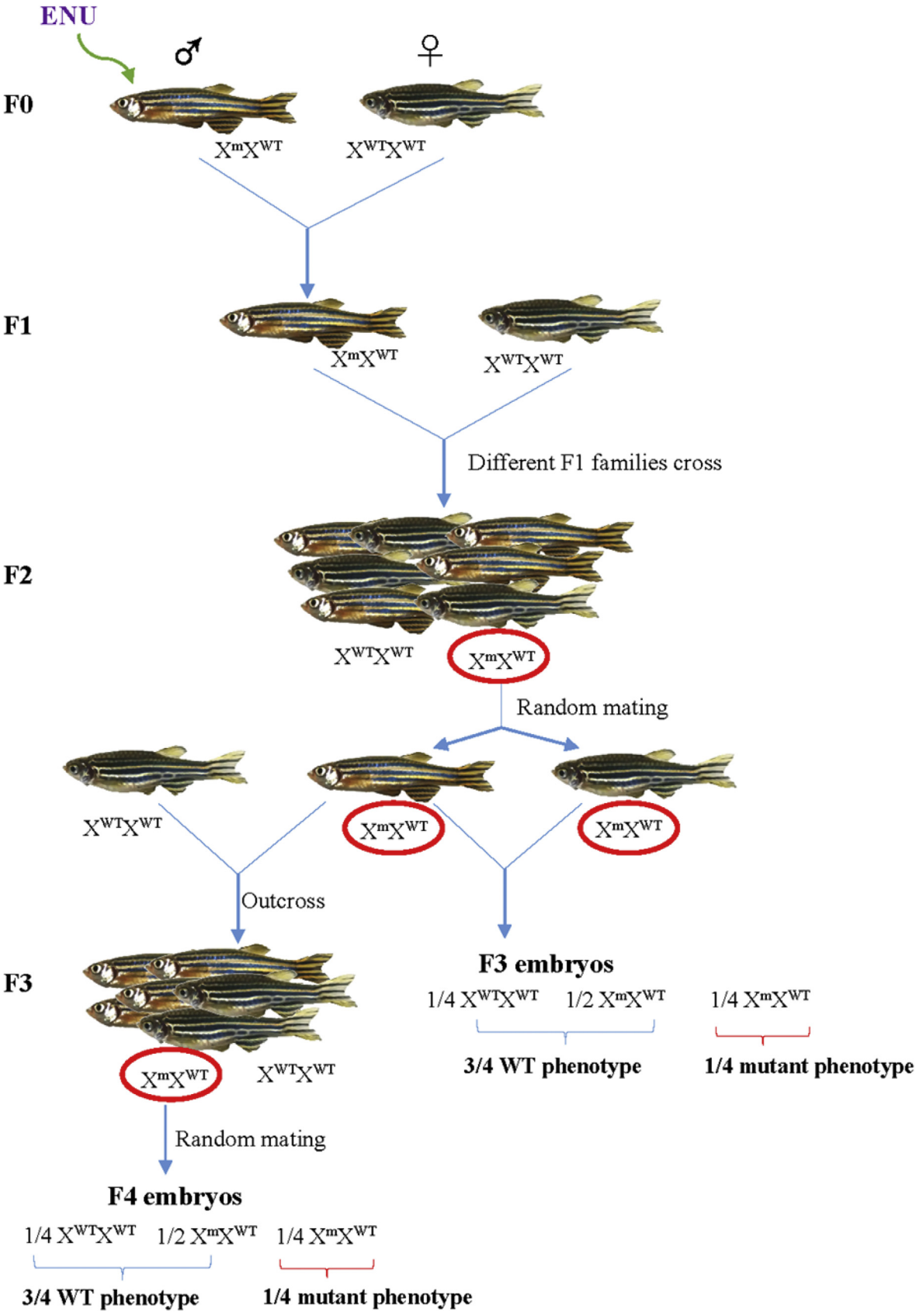

Fig. 1. Breeding and screening in F2 and F3 generation. Thirty male zebrafish were used for ENU treatment. Nine survivors were outcrossed with wild-type fishes to generate F1 families. F2 families were generated by crossing different F1 families (the same mutation locus in one F1 fish is unlikely to occur in another F1). F2 heterozygous mutants were identified by F3 embryos from random in-crossing within each F2 family and outcrossed with wild-type fishes to generate F3 families. Similarly, F4 embryos were used to identify F3 heterozygous mutants.

Table 1
Screening in the F2 generation.

	Number	Rate (%)
Total F2 families	42	100
F2 families identified with mutants	27	64.3
F2 families with F3 families raised	24	57.1
F2 families without mutant or lost	18	42.9
Total crossing pairs	203	100
Putative mutants pairs	52	25.6
Putative mutants pairs given up	9	4.4
Putative mutants pairs for outcross	43	21.2
F3 families raised	34	16.7
Putative mutants lost during maintenance	9	4.4

outcross with wild type fishes while mutants from 3 families were lost during maintenance, F3 families were successfully generated from 34 mutant pairs (from 24 F2 families) (Table 1). Since the F2 families were generated by inter-crossing F1 coming from different F1 families, each F2 family represented two mutagenized genomes. Thus, the scale of our screen had achieved 78 mutagenized genomes.

3.3. Screening in F3 families and complementation experiments

Similar to the screen conducted in F2 families, siblings from F3 families were randomly crossed to obtain F4 embryos. Around 8 crossing pairs were established within each F3 family. Among all the 33 F3 families screened, twenty seven were identified with inheritable phenotypes, one family was given up due to sex bias of fish. A total of 67 mutant pairs originated from 22 F2 families were identified from the WISH outputs of 231 crossing pairs. Mutants with similar phenotypes originating from the same F2 families were selected for complementation experiments to rule out the possibility that they may share the same mutation alleles. Due to the huge amount of workload, allelism tests between different F2 families were not performed. The F3 mutants were finally assigned into 37 lines based on their phenotypes and the original F2 families where they came from (Table 2).

3.4. Classification of the F3 mutant groups

The final 37 F3 mutants were classified into three different groups according to their different phenotypic features (Table 3). Group I is referred to as “intestine specific mutants”. As the name suggests, the mutant intestine show pronounced defect while the liver was less affected (Fig. 2 and supplementary Fig. S1). There were 4 mutants under this group. Group II is defined as “liver specific mutants”, in which the liver exhibited more severe lesions than the intestine (Fig. 3 and supplementary Fig. S2). Two mutants were assigned to this phenotypic group. Group III contains 31 mutants in which both the organs were affected (Fig. 4 and supplementary Fig. S3).

Table 2
Screening in the F3 generation.

	Number	Rate (%)	Number of original F2 families
Total F3 families	33	100	24
Phenotype inheritable	27	81.8	22
Phenotype not inheritable	6	24.2	4
Total crossing pairs	231	100	24
Putative pairs of mutants	67	29.0	22
Candidate mutants with two mutation phenotypes	5	2.2	5

Table 3
Classification of the F3 mutants.

Mutant groups	Number of mutants	Percentage	Phenotypic characteristics	
			Intestine defects	Liver defects
I	4	10.8	√	×
II	2	5.4	×	√
III	31	83.8	√	√

In addition, similar to the other screens, a “Cyclops”-like mutant was identified, in which the embryo body was seriously bended and there was only one eye socket with two retinas (supplementary Fig. S4).

3.5. Organogenesis of the liver and intestine share many common genes

Our screen was designed to examine the status of both the intestine and liver in the mutants generated at the same time in order to study the similarities and differences of the developmental mechanisms between these two distinct organs, albeit less precise due to the scale of the screen. Our results showed that most of our mutants (83.8%) was affected in both organs (Group III), while only a handful demonstrated relatively single defect, with Group I (intestine specific) representing 10.8% and Group II (liver specific) 5.4% (Table 3.). It is cautious to take note that ‘specific’ mutants assigned to Groups I and II did exhibit, though milder, certain defects in the apparently normal liver and intestine respectively. This observation is not totally unpredicted since both endodermal organs develops from an original “intestinal rod” and that the liver develops as an outgrowth of the anterior intestine [23,38]. Therefore only genes that are responsible for cell fate determination and morphogenesis at a specific stage would be expected to exhibit organ specificity, while the earlier mechanisms of mesodermal induction and endodermal differentiation are shared. Since mutations induced by ENU are random and with no gene bias, and our results showed that mutants affected in both organs are the most represented, we can conclude that the majority of the developmental genes are common for both intestine and liver development, and possibly for the whole process of endodermal organogenesis. Indeed, the isolation of liver or intestine specific mutants is rarely reported, putting emphasis in our screening venture.

4. Discussion

4.1. Achievements and analysis of the genetic screen

Zebrafish has long been utilized as a powerful animal model to study the organ development and regeneration. Plenty of ENU mutagenesis screens have been completed in zebrafish, however, screens dedicating to the development of digestive organs is lacking. Unlike other screens, our screen was the first one designed to examine both the intestine and liver at the same time. Moreover, our mutagenesis efficiency is extremely high. In a previous screen performed to identify zebrafish liver mutants, only 19 mutant lines without general defects were isolated from 524 mutagenized genomes. In that effort, the mutagenized efficiency was tested by crossing founders with *albino* mutant and counting *albino* mutant in F1 progenies, reporting an efficiency of 0.2%, which was within the normal mutagenesis rates of 0.09–0.33% ([40], personal communication). In this study, although the mutagenized efficiency was not similarly tested, we

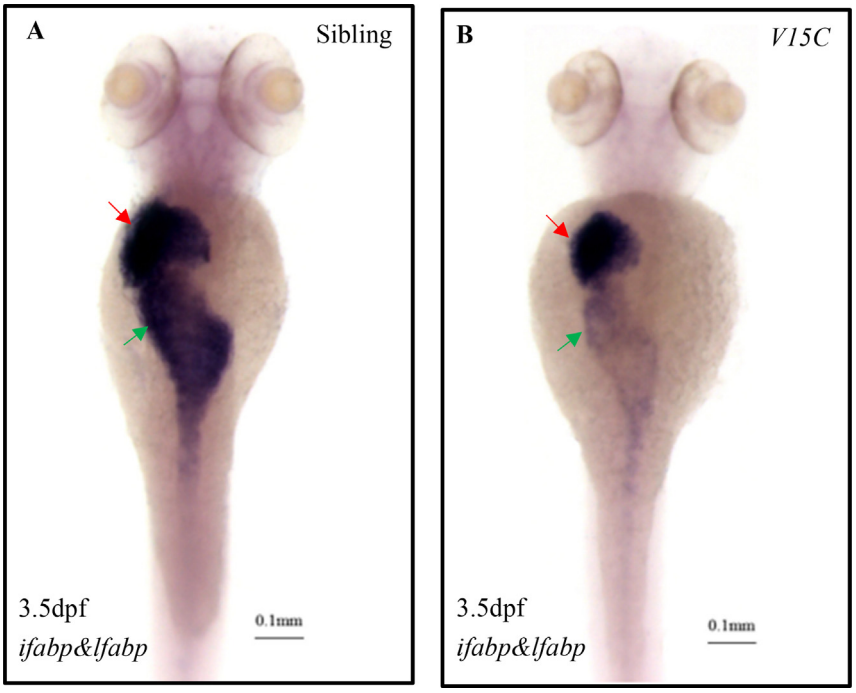

Fig. 2. Representative of Group I mutant. **A** and **B**: WISH experiment of sibling embryos (**A**) and mutant embryos (**B**) at 3.5dpf. Red arrow, the liver, green arrow, the intestine.

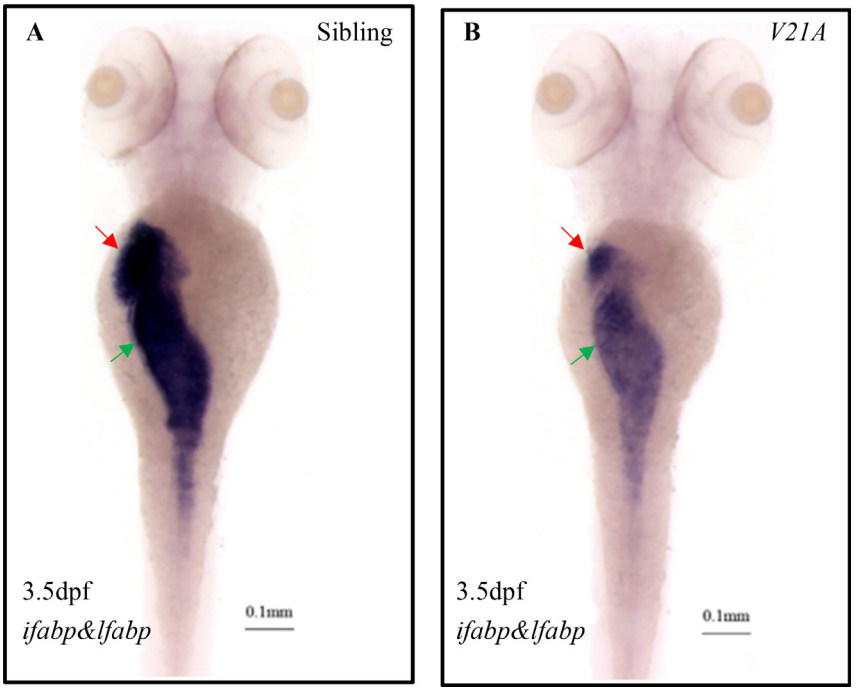

Fig. 3. Representative of Group II mutant. **A** and **B**: WISH experiment of sibling embryos (**A**) and mutant embryos (**B**) at 3.5dpf. Red arrow, the liver, green arrow, the intestine.

obtained 37 putative mutants after screening 78 mutagenized genomes, including 33 liver mutants, indicating a more than 11 fold in efficiency of identifying a liver mutant. To guarantee that the mutant phenotype is heritable, a re-screen was performed in the F3 generation after the classical screen in F2 generation. Five out of the total 67 mutant pairs were found to show double mutation phenotypes in the F3 generation even after one additional round of outcross to segregate multiple mutations. Complementation tests were performed between mutants originated from the same F2 families with similar phenotypes to further exclude the possibility of repeated counting of the same mutations. Among 37 mutants harvested, thirteen F2 families displayed only one mutant phenotype in F3 generation while the rest were identified with multiple mutation types. It is

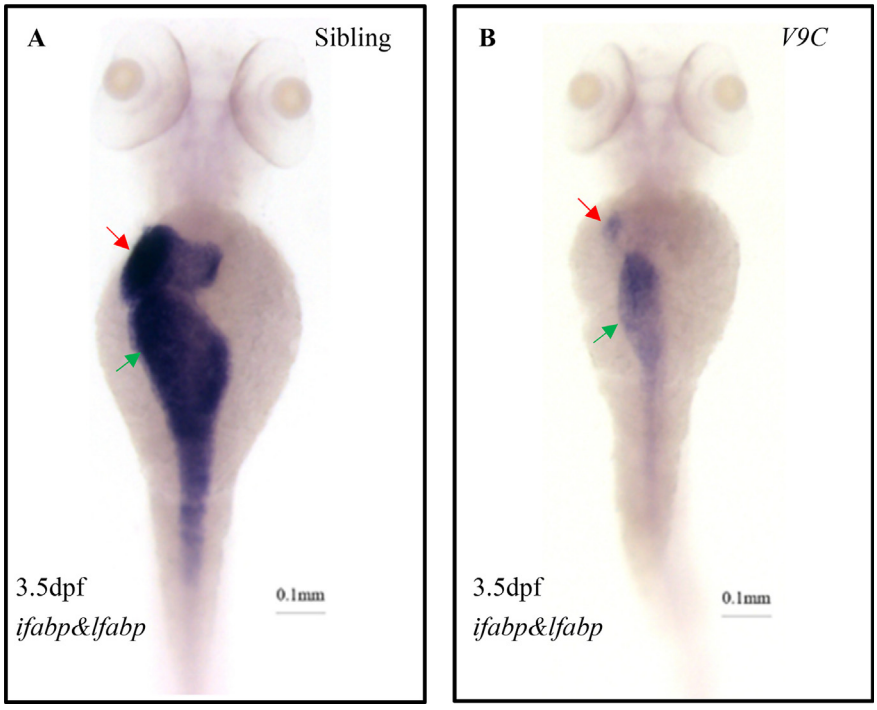

Fig. 4. Representative of Group III mutant. **A** and **B**: WISH experiment of sibling embryos (**A**) and mutant embryos (**B**) at 3.5dpf. Red arrow, the liver, green arrow, the intestine.

noteworthy that there are four F2 families harboring three or four different mutant phenotypes in F3 generation. Based on our data, we can draw the conclusion that the mutation and screening efficiency was considerably high in our protocol.

In summary, a total of 37 mutants were isolated. Through these valuable genetic mutants, it is of our great interest to either identify novel factors or unravel new functions of known genes in digestive system development, of which we believe will contribute greatly to the current understandings of vertebrate digestive organogenesis.

4.2. Limitations of the screen

Restrained by space and manpower, our screen was performed in a limited scale, with only 42 families in total. To overcome these drawbacks, we used F1 in-cross rather than outcross to generate F2 families. This option has enabled optimal space utilization and enhanced our overall screening efficiency. However, with the high mutagenic induction efficiency of ENU, it is unavoidable that inbreeding will lead to a high probability of multiple mutations in progenies, as shown and discussed previously. This consequence can be seen in both our and other screens [28] where the majority of the mutations led to general growth abnormalities resulting in a slow death of the animals. Most of the mutants with general defects were excluded during our screen as our objective is focused on the intestine and liver. It is possible that in our screen, some mutants with potential valuable mutation were aborted, hence missed, due to the presence of another co-existing, lethal mutation, and the undesirable contribution of F1 inbreeding to generate F2 to this outcome should not be underestimated.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.119.

Transparency document

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