Degenerative diseases of organs lead to their impaired function. The cellular and molecular mechanisms underlying organ degeneration are therefore of great research and clinical interest but are currently incompletely characterized. Here, using a forward-genetic screen for genes regulating liver development and function in zebrafish, we identified a cg5 mutant that exhibited a liver-degeneration phenotype at 5 days postfertilization, the developmental stage at which a functional liver develops. Positional cloning revealed that the liver degeneration was caused by a single point mutation in the gene zc3h8 (zinc finger CCCH-type containing 8), changing a highly conserved histidine to glutamine at position 353 of the Zc3h8 protein. The zc3h8 mutation—induced liver degeneration in the mutant was accompanied by reduced proliferation, increased apoptosis, and macrophage phagocytosis of hepatocytes. Transcriptional profile analyses revealed up-regulation and activation of both proinflammatory cytokines and the NF-κB signaling pathway in the zc3h8 mutant. Suppression of NF-κB signaling activity efficiently rescued the proinflammatory cytokine response, as well as the inflammation-mediated liver degeneration phenotype of the mutant. Of note, the zc3h8 mutation-induced degeneration of several other organs, including the gut and exocrine pancreas, indicating that Zc3h8 is a general repressor of inflammation in zebrafish. Collectively, our findings demonstrate that Zc3h8 maintains organ homeostasis by inhibiting the NF-κB–mediated inflammatory response in zebrafish and that Zc3h8 dysfunction causes degeneration of multiple organs, including the liver, gut, and pancreas.

The liver is a crucial digestive organ with a central role in metabolic homeostasis. Hepatocytes constitute the majority of the liver and perform several critical functions, including the production of bile, storage of glycogen, detoxification, and regulation of blood homeostasis (1–3). Degenerative diseases involve a continuous process of cell degeneration, which affects tissue or organ function, with increasing deterioration occurring over time. Nonreversible damage to liver function results in the death of the affected organism. Thus, a better understanding of the molecular mechanisms involved in liver degeneration is essential for the development of novel drug therapies and cures for these diseases.

Inflammation is triggered by microbial pathogens or danger signals derived from the host. The receptors of antigen-presenting cells sense microbial products (4) and endogenous signals released by damaged cells (5), leading to their activation, and subsequently initiate an inflammatory response. Previous studies (6, 7) have shown that inflammation is a major cause of most chronic liver diseases, and inhibition of the activation of inflammasomes (multiprotein complexes that recognize danger signals from damaged cells and pathogens (8)) could ameliorate inflammation-related diseases. The transcription factor NF-κB is a major factor that mediates and regulates the expression of many genes involved in inflammation (9). Inactive NF-κB is located in the cytoplasm. Once it becomes phosphorylated and activated, it translocates to the nucleus, where it binds to the promoters of specific target genes and can up-regulate the transcription of cytokines and chemokines.

Zebrafish have been recognized as a powerful model organism for the study of liver development and regeneration because of their embryological and genetic advantages (10–14). In this study, we used forward genetic screening to identify genes that regulate liver development and regeneration in zebrafish. Interestingly, we found that the cg5 mutant exhibited severe liver degeneration defects at later stages of development (after 5 days postfertilization (dpf)) and a shorter life span. Positional cloning revealed that the zinc finger protein Zc3h8 (zinc finger CCCH-type containing 8) was mutated and contributed to the cg5 mutant degenerative phenotype. Further
analysis showed that liver degeneration in the cq5 mutant was accompanied by a proinflammatory response and activation of the NF-κB pathway, whose inhibition efficiently rescued hepatocyte degeneration in the mutant. These findings suggest a repressor role of Zc3h8 in liver homeostasis during the inflammatory response in zebrafish and may provide a new avenue for research on and treatment of degenerative diseases in humans.

Results

The cq5 mutant exhibits liver degeneration

Abnormal liver development of the cq5 mutant was identified in a zebrafish N-ethyl-N-nitrosourea forward genetic screen under the Tg(fabp10a:Dendra2-NTR) line (10) for genes regulating liver development and regeneration. Homozygous cq5 mutants did not show any observable phenotype before 4 dpf (Fig. 1, A, A’, B, and B’) but exhibited a large amount of unabsorbed yolk at 5 dpf (Fig. 1, C and C’). Survival curves showed that the survival rate of the mutants decreased dramatically from 6 dpf on, with all the mutants dead at 13 dpf (Fig. 1E).

At 4 dpf, the liver of the mutant remained normal (Fig. 1, F and F’). From 4 to 5 dpf, although the mutant liver continued growing, its outgrowth rate was slower than the WT fish (Fig. 1, F, F’, G, and G’). The cq5 mutant subsequently exhibited degeneration of hepatocytes (Fig. 1, H, H’, I, and I’), displaying a one-third volume of the WT liver at 8 dpf (Fig. 1J). These results demonstrate that mutation of the cq5 mutation is ineffective to the early liver development but does impair liver growth and homeostasis, thus leading to liver degeneration after 4 dpf.

The cq5 mutant phenotypes are caused by the mutation of zc3h8 encoding a CCCH-type zinc finger protein

Positional cloning identified that the mutation was localized in three candidate genes, fbln7, zc3h8, and itga9, flanked by the markers 1006 and 1023 on the linkage group 13 (LG13) (Fig. S1, A and B). Through sequence analyses of these three candidate cDNAs, we identified a single point mutation in zc3h8 (Fig. 2A). This C-to-G transition generated an amino acid switch from histidine to glutamine at position 353 (Fig. 2A). Structurally, the results of syntenic analyses and homologous protein sequence alignment of Zc3h8 in different species (Fig. S1, B and C) revealed that the proteins in all species contained three conserved CCCH-type functional domains, and the mutated histidine in the cq5 mutant was evolutionarily conserved (Fig. 2B).

To validate that the cq5 liver degeneration phenotype was caused by the zc3h8 mutation, we generated a Tg(hsp70l:HA-zc3h8-T2A-mCherry) transgenic line (hereafter referred to as hszc3h8) (Fig. S2A), which permitted visualization of red fluorescence after heat shock. Heat shock–induced overexpression of Zc3h8 in the cq5 mutant efficiently rescued the degenerated liver at 6.5 dpf (Fig. S2, B and C). Furthermore, hepatocyte-specific replenishment of WT Zc3h8 using the Tg(fabp10a:HA-zc3h8-T2A-mCherry) line (referred to as fabp10a:zc3h8) transgenic background also efficiently rescued the degenerated liver in the cq5
mutant (Fig. S2, D–H). These results confirm that the liver phenotypes of the cq5 mutant caused by the zc3h8 mutation.

Further confirmation was carried out using the transcription activator-like effector nuclease (TALEN) technology (23) to generate a zc3h8 mutant. Exon 7 was selected as the target of TALEN nuclease, where the cq5 mutation is located (Fig. S3A). Two independent zc3h8 mutant alleles were generated. One allele harbored a 3-bp deletion and a 8-bp insertion (referred to as zc3h8inscq45), leading to disrupted protein sequence from amino acid 351 and a premature stop codon at amino acid 382. The other allele harbored a 10-bp deletion (referred to as zc3h8/H900410cq46), leading to disrupted protein sequence from amino acid 351 and a premature stop codon at amino acid 377 (Fig. S3, B and C). Both zc3h8inscq45 and zc3h8Δ10cq46 showed liver degeneration at 6.5 dpf, similar to the cq5 mutant (Fig. S3, D–G). These data validate that the liver degeneration phenotype of cq5 mutant is caused by the zc3h8 mutation.

To determine whether the histidine to glutamine switch of Zc3h8 affects the protein stability, the heat shock promoter-driven HA-zc3h8H353Q-T2A-mCherry plasmid that contained the same mutation as the cq5 mutant, as well as the WT HA-zc3h8-T2A-mCherry plasmid, were generated. Similar expression levels of the WT and mutated proteins (Fig. 2C) indicated that the point mutation was ineffective to protein stability of Zc3h8. 

In situ hybridization and qPCR of zc3h8 showed strong expressions in the brain and eyes and relatively weak expressions in the digestive system at 96 hpf (Fig. 2, D–F). Fluorescent in situ hybridizations (FISHs) confirmed the expression of zc3h8 in hepatocytes (Fig. 2G). These data indicate that zc3h8 is required for the maintenance of liver homeostasis.
Roles of Zc3h8 in digestive organ degeneration

Zc3h8 mutant liver degeneration is accompanied by reduced proliferation and increased apoptosis of hepatocytes

The balance of cell proliferation and apoptosis affects tissue development and determines organ size (24). Many previously reported mutants exhibiting smaller livers show low proliferation of hepatocytes (25–27). To determine whether the liver-degenerative phenotype of the \( \text{cq5} \) mutant was the result of abnormal cell proliferation or not, EdU incorporation ratios were analyzed. The proliferation rates of mutant hepatocytes remained similar to the control at 4.5 dpf (Fig. 3, A, A’, A”, B, B’, and B”) but were significantly reduced at 5.5 dpf (Fig. 3, C, C’, D, D’, and K).

Macrophages play important roles in inflammation, recognition, and phagocytosis of apoptotic cells, as well as tissue repair and regeneration (28). To investigate roles of macrophages in the liver degeneration process, the \( \text{Tg(fabp10a:mCherry-NTR)}^{\text{m2}} \) and \( \text{Tg(mpeg1:EGFP)} \) transgenic backgrounds were applied. Macrophage recruitment to the mutant liver and engulfment of hepatocytes became observed at 5 dpf (Fig. 3, E, and I–I”).

Figure 3. Reduced proliferation and increased apoptosis of hepatocytes are associated with \( \text{zc3h8} \) mutation. A–D and A’–D”, confocal images of EdU and anti-Dendra2 antibody labeling in WT and mutant larvae from 4.5 (29 of 29, 25 of 27) to 5.5 dpf (28 of 29, 25 of 28). Scale bars, 20 \( \mu \)m. E–H and E’–H’, \( \text{Tg(fabp10a:mCherry-NTR); mpeg1:EGFP)} \) double-transgenic line fish were visualized via confocal microscopy from 5 (E–E’ and F–F’; 53 of 53, 48 of 52) to 6.5 dpf (G–G’ and H–H’; 54 of 54, 55 of 59) for WT and \( \text{zc3h8} \) mutant larvae. White arrowheads highlight macrophages engulfing degenerated hepatocytes. Scale bars, 20 \( \mu \)m. I, I’, J, and J’, confocal images of TUNEL-labeled apoptotic cells in \( \text{zc3h8} \) mutant and WT livers at 5.5 dpf (38 of 40, 42 of 45). Scale bars, 50 \( \mu \)m. K, quantification of EdU-labeled hepatocytes in the \( \text{zc3h8} \) mutant and the WT at 4.5 dpf (n = 5, n = 9) and 5.5 dpf (n = 8, n = 8). The error bars indicate S.D. NS represents no significance. ***, \( p < 0.001 \) by Student’s t test. L, quantification of EdU-positive hepatocytes in \( \text{zc3h8} \) mutant and WT (n = 12) larvae at 5.5 dpf. The error bars indicate S.D. ***, \( p < 0.001 \) by Student’s t test. M, quantification of TUNEL-positive hepatocytes in \( \text{zc3h8} \) mutant and WT larvae at 5 dpf (n = 10, n = 10) and 6.5 dpf (n = 9, n = 10). The error bars indicate S.D. ***, \( p < 0.001 \) by Student’s t test.
Roles of Zc3h8 in digestive organ degeneration

Expressions of digestive organ markers (flabp10a, insulin, trypsin, and ifabp) indicated normal liver, pancreas, and gut at 3 dpf (Fig. S5A). At 4 dpf, expressions of trypsin and ifabp started to become weaker in the mutant than in the WT (Fig. S5B). Applications of the Tg(mpeg1:EGFP), Tg(cdih17:EGFP), and Tg(p48:GFP) transgenic backgrounds revealed degenerations of gut and exocrine pancreas from 5 to 7 dpf (Fig. S5, C, D, E–E’, F–F’, G–G’, and H–H’). The degenerated pancreas was partially rescued by the treatment of Dex or JSH-23 (Fig. S6, A and B), suggesting that degenerations of other digestive organs caused by zc3h8 deficiency involve mechanisms similar to liver degeneration. To investigate whether Zc3h8 could act as a general repressor of inflammation, we microinjected Escherichia coli into the circulation at 2 dpf to induce the inflammatory response (35). qPCR analysis indicated that the highly expressed pro-inflammatory cytokines were partially rescued by Zc3h8 overexpression (Fig. S6C), suggesting Zc3h8 as a general repressor of inflammation.

Discussion

Declining organ function is a common disease (36, 37), and research on this topic has important implications for human health. CCCH-type zinc finger proteins are important members of the zinc finger family proteins (18, 22, 38). Many previous studies on these proteins have been limited to mammals, and almost no studies have described the functions of CCCH-type zinc finger proteins or the phenotypes of mutants in zebrafish. This report is the first to identify a liver defect-related mutant, cq5, harboring a mutation in zc3h8, which encodes a typical zinc finger protein containing three conserved CCCH-type domains. This mutation resulted in degeneration of the zebrafish liver, gut, and pancreas. Further investigation revealed roles of Zc3h8 in maintaining hepatocyte metabolism through inhibition of proinflammatory responses in zebrafish.

To determine the function of the zc3h8 gene in zebrafish, the results of systemic gene analysis and homologous protein sequence alignment were evaluated, which indicated that zc3h8 of fish may exhibit the conserved functions of zc3h6 and zc3h8 in mammals (humans and mice). However, no clear function has been reported for zc3h6 in humans and mice. Another homologous gene of zc3h8 in mice (fetal liver zinc-finger protein 1, Fliz1) has been reported to be expressed in the fetal mouse liver (39) and plays a role in regulating thymocyte homeostasis (40). Additionally, overexpression of fliz1 represses the expression of gata3 (GATA-binding protein 3) (41). However, the expression of gata3 does not differ between WT and zc3h8-overexpressing zebrafish (data not shown). This observation indicates that zc3h8 of zebrafish may have a different function than fliz1 in the mouse.

E’, F, and F’), which were significantly increased at 6.5 dpf (Fig. 3, G, G’, H, H’, and L). TUNEL assays showed that apoptotic hepatocytes significantly increased in the mutant at 5.5 dpf (Fig. 3, I, I’, J, J’, J’, and M). These data suggest that liver degeneration in the cq5 mutant is contributed by both of the decreased hepatocyte proliferation and increased hepatocyte apoptosis.

Blockade of inflammation and NF-κB signaling rescue liver degeneration in the mutant

To explore the regulatory molecules and signaling pathways involved in mutant liver degeneration, we dissected the liver and sorted the Dendra2-positive hepatocytes at two different stages (middle stage at 5.5 dpf and late stage at 7.5 dpf) using the Tg(fabp10a:Dendra2-NTR)αβ transgenic background (Fig. 4A). Transcriptional profile analyses revealed up-regulation of inflammation-related molecules in the mutant hepatocytes at both middle and late degenerative stages (Fig. 4B). qPCRs verified that expressions of hepatocyte-specific markers (p2, tfa, and ttr) significantly decreased in the zc3h8 mutant (Fig. 4C), whereas expressions of inflammation-related genes increased at both 5.5 and 7.5 dpf (Fig. 4D). These data demonstrated activation of inflammatory responses in the mutant. Treatment of anti-inflammatory drug dexamethasone (Dex) from 4 to 6 dpf partially rescued liver degeneration in the mutant (Fig. 4, E–H), suggesting the inflammatory response as one of the inducers of liver degeneration.

NF-κB plays important roles in innate and adaptive responses, inflammation, proliferation, and cellular differentiation (29–31). Inactive NF-κB is located in the cytoplasm. Once it is phosphorylated and activated, NF-κB translocates into the nucleus and up-regulates the transcription of cytokines and chemokines (32, 33). To investigate whether the inflammation-induced liver degeneration is mediated by the NF-κB activities, expressions of the active NF-κB components nfkbiaa and nfkbibab were first analyzed. Transcriptional activations of nfkbiaa and nfkbibab were undetectable at 4.5 dpf but became significantly increased at 5.5 dpf (Fig. 5, A, A’, B, B’, C, C’, D, and D’). To obtain additional evidences for the increased presence of activated NF-κB in degenerating hepatocytes, immunostainings of the active NF-κB component, phosphorylated p65 (S276-p65), were performed. Although the hepatic S276-p65 signals in the mutant at 4 dpf remained similar to the WT, nuclear accumulation of S276-p65 steadily increased from 4 to 6 dpf in the mutant liver (Fig. S4, A–F and A’–F’, and Fig. 5, G and G’). Because the number of apoptotic hepatocytes remained unchanged in the mutant at 4.5 dpf (Fig. S4, G–M), activation of the NF-κB signaling occurred prior to the hepatocyte apoptosis in the mutant.

Blockade of NF-κB signaling using a chemical inhibitor JSH-23 (34) reduced the nuclear accumulation of S276-p65 (Fig. 5, F–H and F’–H’), resulting in partial rescue of degenerated liver and even full rescue of the increased hepatocyte apoptosis in the mutant (Figs. 5, I–M, and 6, A–E). The reduced proliferation of mutant hepatocytes was not rescued by the treatment of JSH-23 (Fig. 6, F–J). JSH-23 treatment was ineffective to the activation of NF-κB component nfkbibaa in the mutant (Fig. 7, C, C’, D, and D’). However, activations of il1b and mpx at 6.5 dpf in the mutant were efficiently repressed by the JSH-23 (Fig. 7, G, G’, H, H’, K, K’, L, and L’). These data indicate that the NF-κB-mediated inflammation acts as one of the inducers of liver degeneration caused by zc3h8 deficiency.

Zc3h8 acts as a general inflammation repressor primarily through NF-κB signaling

Activations of nfkbibaa and nfkbibab were also observed in the gut of the cq5 mutant (Fig. 5, B’ and D’), implicating that the degeneration phenotype is not only restricted to the liver. Expressions of digestive organ markers (flabp10a, insulin, trypsin, and ifabp) indicated normal liver, pancreas, and gut at 3 dpf (Fig. S5A). At 4 dpf, expressions of trypsin and ifabp started to become weaker in the mutant than in the WT (Fig. S5B). Applications of the Tg(mpeg1:EGFP), Tg(cdih17:EGFP), and Tg(p48:GFP) transgenic backgrounds revealed degenerations of gut and exocrine pancreas from 5 to 7 dpf (Fig. S5, C, D, E–E’, F–F’, G–G’, and H–H’). The degenerated pancreas was partially rescued by the treatment of Dex or JSH-23 (Fig. S6, A and B), suggesting that degenerations of other digestive organs caused by zc3h8 deficiency involve mechanisms similar to liver degeneration. To investigate whether Zc3h8 could act as a general repressor of inflammation, we microinjected Escherichia coli into the circulation at 2 dpf to induce the inflammatory response (35). qPCR analysis indicated that the highly expressed pro-inflammatory cytokines were partially rescued by Zc3h8 overexpression (Fig. S6C), suggesting Zc3h8 as a general repressor of inflammation.
Roles of Zc3h8 in digestive organ degeneration

Figure 4. zc3h8 mutant hepatocyte degeneration is inflammatory response–dependent. A, schematic representation of the process of transcriptome sequencing or qPCR analysis of sorted hepatocytes and direct qPCR analysis of liver tissues from WT and zc3h8 mutants. B, heat map of the log10-transformed expression of statistically significant, differentially expressed genes between sorted hepatocytes of WT and zc3h8 mutants. Red indicates up-regulation, and blue indicates down-regulation of mRNA compared with WT. C, quantitative real-time PCR of hepatocyte-specific markers for sorted hepatocytes from zc3h8 mutant and WT larvae at 6.5 dpf. The error bars represent S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001 by Student’s t test. D, quantitative real-time PCR analysis of proinflammatory cytokine expression in liver tissues at 5.5 and 7.5 dpf. The error bars represent S.E. ns represents no significance. *, p < 0.05; **, p < 0.01; ***, p < 0.001 by Student’s t test. E–G, fluorescence confocal microscopy of Tg(fabp10a:Dendra2-NTR) in zc3h8 mutant (31 of 44) and WT (49 of 56) livers treated with Dex or methanol (56 of 56) at 6.5 dpf. Note that the zc3h8 mutant degeneration phenotype could be partially rescued by treatment with the anti-inflammatory drug Dex. Scale bars, 50 μm. H, quantification effects of Dex on zc3h8 mutant degeneration liver at 6.5 dpf. The error bars represent S.D. ***, p < 0.001 by Student’s t test.
All CCCH-type zinc finger proteins function through the characteristic CCCH zinc finger domain. The cq5 mutation site is located in the second CCCH zinc finger domain, where an amino acid coding change from histidine to glutamine occurs.

We produced two lines of TALEN-induced knockout fish, zc3h8ins5cq45 and zc3h8/H900410cq46 (not containing the third CCCH zinc finger domain-truncated proteins), both of which showed the same degeneration phenotype as the cq5 mutant.

Although we did not determine the molecules that could directly bind to the CCCH domain, these results suggest the importance of the second and third CCCH zinc finger domains for the function of the Zc3h8 protein.

In our analysis of the mutant phenotype, we noted that cq5 mutant larvae exhibited gradual digestive organ loss and degradation after 4 dpf, especially for the liver. However, the expression levels of organ markers and morphological phenotypes before 4 dpf were comparable between the mutant and WT larvae. The relatively stable expression of zc3h8 in hepatocytes demonstrated by FISH analysis could likely explain the degenerative phenotype observed in the mutant liver, and hepatocyte specific expression of Zc3h8 could absolutely rescue the liver defect of zc3h8 mutant (Figs. S2, D–H, S7E) and partly reduce the proinflammatory response (Fig. S7, F–H). Although the other defect phenotypes of gut, pancreas, retina, swim bladder, and brain not rescued by the liver overexpression Zc3h8 (Fig. S7, A–D), this indicated that the degeneration process of the mutant hepatocytes was autonomous and independent of the other type cells induced.

Our transcriptome analysis revealed that NF-κB signaling and inflammatory cytokines were both up-regulated during...
liver degeneration. This finding is consistent with previous studies showing that NF-κB signaling–mediated inflammatory responses are important for liver homeostasis and wound healing (42–45). At the RNA level, in situ hybridization of NF-κB component genes and qPCR analysis of inflammatory cytokine levels showed increased expression of NF-κB signaling-related genes and inflammatory cytokines in the degenerated mutant liver. At the protein level, the results of antibody staining for phosphorylated p65 (RelA) combined with treatment using inhibitors indicated that blocking the NF-κB signal or inflammatory response could rescue the mutant phenotype. Further confirming the Zc3h8 role as a repressor of inflammatory response, bacteria-induced inflammatory model was established, and its high expression of inflammation cytokines could be partially rescued by the overexpression of Zc3h8 (Fig. S6C). These observations are consistent with studies analyzing other CCCH-type zinc finger proteins, such as TTP (18, 21, 22, 46).

In summary, although the zc3h8 mutant exhibited the non-liver specific abnormal phenotype, but our results indicated the degeneration process and mechanism in the liver were similar with the other digestive organs. Our work reveals that Zc3h8 plays important roles in maintaining digestive organs homeostasis, and mutation of this gene causes their degeneration. Mechanistically, this degeneration process depends on the NF-κB pathway and related inflammatory responses. This study expands our understanding of CCCH-type zinc finger protein function in protecting digestive system cells including the hepatocytes from degeneration and maintaining organ development in zebrafish and provides new insight for future research and the development of degenerative organ disease therapeutics.

**Experimental procedures**

**Ethics statement**

All experimental protocols were approved by the School of Life Sciences, Southwest University (Chongqing, China), and the experiments were carried out in accordance with the approved guidelines. The zebrafish facility and study were approved by the Institutional Review Board of Southwest University (Chongqing, China). Zebrafish were maintained in...
accordance with the Guidelines for Experimental Animal Welfare from the Ministry of Science and Technology of the People’s Republic of China (2006) and the Institutional Animal Care and Use Committee protocols of Southwest University (2007).

Zebrafish lines and embryo culture

Adult fish and embryos were raised and maintained under standard laboratory conditions according to Institutional Animal Care and Use Committee protocols of Southwest University (2007).

Figure 7. Uncontrolled inflammation is induced by activated NF-κB signaling. A–D and A’–D’, whole-mount in situ hybridization of an NF-κB component gene (nfkbia) in WT and zc3h8 mutant larvae at 5.5 and 6.5 dpf after pretreatment with DMSO or JSH-23. Red arrowheads point to the liver, and the arrows point to the intestine. E–L and E’–L’, whole-mount in situ hybridization of proinflammatory cytokines (il1b and mpx) in WT and zc3h8 mutant larvae at 5.5 and 6.5 dpf after pretreatment with DMSO or JSH-23. Red arrowheads point to the liver, and the black arrows point to the intestine.

Roles of Zc3h8 in digestive organ degeneration

Heterozygous cq5 fish were crossed with the polymorphic line SJD to generate the mapping population. A total of 240 transgenic lines were used: Tg(fabp10a:Dendra2-NTR)<sup>q1</sup>, Tg(fabp10a:mCherry-NTR)<sup>q2</sup>, Tg(sox17:GFP)<sup>q870</sup>, Tg(p48:GFP), Tg(cdhl7:EGFP), and Tg(mpeg1:EGFP). Fish embryos were treated with 0.003% 1-phenyl-2-thiourea (Sigma–Aldrich) from 24 hpf to inhibit pigmentation.

Positional cloning of the cq5 mutation gene

Previously described (47). The following transgenic lines were used:
Roles of Zc3h8 in digestive organ degeneration

Table 1

| Target   | Forward (5’→3’)                      | Reverse (5’→3’)                  | Used for  |
|----------|--------------------------------------|----------------------------------|-----------|
| 1006     | CCATACCGTGACCCACCTGCTA              | TCTCAGCTGAGCCTCTCTCTAC           | PCR       |
| 1023     | GCCACATTGTCACCAAAAGCT              | TACTGGCTGACCACCTCTCAC           | PCR       |
| zc3h8-exon7 | AAAGTCCACACCTGGTGTACCA          | AGGCCTGGAGCAGGTTTCTCC          | PCR       |
| zc3h8-HA | AGTATCCATACGGTATTCCTCCTGGGTGATCCCT | GGGTGCTTATTTGGAGATTGA          | PCR       |
| zc3h8-L  | GACATCCATACGGTATTCCTCCTGGGTGATCCCT | GGACTGACAGTATGGCATGTATTTGCT   | PCR       |
| zc3h8-R  | GACATCCATACGGTATTCCTCCTGGGTGATCCCT | GTTCATGACAGTATGGCATGTATTTGCT   | PCR       |
| T2A-mcherry | CCATACCGTGACCCACCTGCTA         | TCTCAGCTGAGCCTCTCTCTAC           | PCR       |
| f2      | CTTGATGGATGCTGTCG                | GCTGCTGTTGAGTCCTCTC             | qPCR      |
| ifa     | TCTGCTGCTGCTGCACTGCCGCTGG        | GATCTCGCTGCTGCACTGCCGCTGG       | qPCR      |
| irr     | CCTTGCTGCTGCTGCTGCTGCTGCTG      | GTACCTCGCTGCTGCTGCTGCTGCTG     | qPCR      |
| i220    | ATGCTGCTGCTGCTGCTGCTGCTGCTG     | GTACCTCGCTGCTGCTGCTGCTGCTG     | qPCR      |
| i1b     | ATGACCCACCCACCTGCTGCTGCTG         | GGACTGACTGACTGACTGACTGACTGACTG | qPCR      |
| il6     | GACATCCATACGGTATTCCTCCTGGGTGATCCCT | GTTCATGACAGTATGGCATGTATTTGCT   | PCR       |
| il8     | GACATCCATACGGTATTCCTCCTGGGTGATCCCT | GTTCATGACAGTATGGCATGTATTTGCT   | PCR       |
| mnx     | CTTGATGGATGCTGTCG                | GCTGCTGTTGAGTCCTCTC             | qPCR      |
| sfa     | GACATCCATACGGTATTCCTCCTGGGTGATCCCT | GTTCATGACAGTATGGCATGTATTTGCT   | PCR       |
| sfb     | GACATCCATACGGTATTCCTCCTGGGTGATCCCT | GTTCATGACAGTATGGCATGTATTTGCT   | PCR       |
| fabp10a | CTTGATGGATGCTGTCG                | GCTGCTGTTGAGTCCTCTC             | qPCR      |
| b-actin | TCTGCTGCTGCTGCTGCTGCTGCTGCTG     | GTACCTCGCTGCTGCTGCTGCTGCTG     | qPCR      |
| zc3h8   | GACATCCATACGGTATTCCTCCTGGGTGATCCCT | GTTCATGACAGTATGGCATGTATTTGCT   | qPCR      |
| mnb     | GTTCAATGTCGACCCACCTGCTGCTG       | AATCGATGCGATGCTGCTGCTGCTG       | qPCR      |
| nfbkiaa | AGTTCAATGTCGACCCACCTGCTGCTG     | AATCGATGCGATGCTGCTGCTGCTG       | WISH      |
| nfbkiab | ATGACCCACCCACCTGCTGCTGCTG         | GGACTGACTGACTGACTGACTGACTGACTG | WISH      |
| zc3h8   | GACATCCATACGGTATTCCTCCTGGGTGATCCCT | GTTCATGACAGTATGGCATGTATTTGCT   | qPCR      |
| insulin | GCTGGATGCTGCTGCTGCTGCTGCTGCTG     | GTACCTCGCTGCTGCTGCTGCTGCTG     | qPCR      |
| fabp10a | CTTGATGGATGCTGTCG                | GCTGCTGTTGAGTCCTCTC             | qPCR      |
| ifabp   | GACATCCATACGGTATTCCTCCTGGGTGATCCCT | GTTCATGACAGTATGGCATGTATTTGCT   | PCR       |
| Mpx     | CCTACTGCTGCTGCTGCTGCTGCTG       | AATCGATGCGATGCTGCTGCTGCTG       | WISH      |
| illb    | CCATACCGTGACCCACCTGCTA              | TCTCAGCTGAGCCTCTCTCTAC           | PCR       |

SSLP (simple sequence length polymorphism) Luo lab Z markers were used in bulked segregant analysis. The amplification reactions of positional cloning were performed with 2× Taq Master Mix (Novoprotein, Shanghai, China). SSLP markers z6657 and z30283 from chromosome 13 were found to be linked to the cz5 mutation. After testing 560 mutant embryos, the location of the cz5 mutation was further narrowed to between z55656 (14 recombinants) and z30283 (47 recombinants). New SSLP markers were designed. Two markers, 1006 and 1023, identified three and two recombinants, respectively, within genomic DNA fragments of 0.31 million bp, containing three genes (jfbn7, zc3h8 and itga9). The mutant genotype was determined by sequencing the PCR fragment containing the mutated bases.

Zc3h8 TALEN assembly, targeting, and allele analysis

A TALEN targeting zc3h8 exon7 was designed using TAL effector Nucleotide Targeter 2.0 (TALE-NL 2.0) (48) and was assembled using the modified Golden TALEN scaffold (49). After mixing the pair of target-site TALEN mRNAs, a final concentration of ~150–200 pg was injected into one-cell-stage WT embryos. Genomic DNA was subsequently extracted from the embryos, and the PCR products were sequenced. The primers used for amplification of the zc3h8 exon7 are listed in Table 1; a 524-bp DNA fragment containing the mutant target site was amplified via PCR and sequenced. After the embryos were grown to maturity, they were outcrossed to identify F1 progeny in which infidelity during repair by nonhomologous end joining introduced indels at the cleavage site that could disrupt the zc3h8 reading frame.

Construction of the zc3h8-overexpressing transgenic plasmid and lines

To generate the Tg(hsp70l:HA-zc3h8-T2A-mCherry)z30283 and Tg(fabp10a:HA-zc3h8-T2A-mCherry)z30283 transgenic lines of zebrafish, we first constructed the transgenic hsp70l:HA-zc3h8-T2A-mcherry plasmid (Fig. S8A). An HA-tag–encoding sequence was incorporated into the forward primer (Table 1). The full-length cDNA of zebrafish zc3h8 (XM_682645) was amplified from 24-hpf ABGO cDNA using high-fidelity PrimeSTAR DNA polymerase (TaKaRa, Shiga, Japan), and the PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI) to generate pGEMT-HA-zc3h8. Positive clones were subsequently picked and sequenced. Next, we amplified fragments with no mutations in the HA-zc3h8 fragment and inserted them into the pDsRed2- vector, which had a Kana-resistant backbone, to generate pDsRed2-HA-zc3h8 by replacing DsRed2 via Nhel/NotI digestion. We separately amplified the left and right arms (~500 bp) of the HA-zc3h8 fragment from the pGEMT-HA-zc3h8 and T2A-mcherry fragments from the pLEntiloxx3.7 shRNA GFP vector plasmid. Two cycles of overlapping PCR were performed to generate HA-zc3h8 arm-T2A-mcherry, which was then cloned into the hsp70l:Cre plasmid after the excision of Cre with BamHI/NotI. Recombineering was accomplished via the following steps. We transformed a mixture of plasmid pDsRed-HA-zc3h8 with linearized hsp70l:HA-zc3h8 arm-T2A-mcherry fragments into electrocompetent SW102 cells, which can produce the recombinase used for the recombination reaction, to generate hsp70l:HA-zc3h8-T2A-mcherry. The HA-zc3h8-T2A-mcherry fragment was cloned into fabp10a:Dendra2NTR plasmid after excision of Dendra2NTR with Nhel/NotI to generate fabp10a:HA-zc3h8-T2A-mcherry. The plasmids were co-injected with I-SceI meganuclease into...
Roles of Zc3h8 in digestive organ degeneration

Cell sorting, transcriptome sequencing, and quantitative real-time PCR

Approximately 150–200 transgenic Tg(fabp10a:Dendra2-NTR)cq43 larvae at 5.5 or 7.5 dpf were dissected, and their hepatocytes were then dissociated and sorted as previously described (12). The Dendra2-positive hepatocytes separated from the control or sample groups were subjected to transcriptome sequencing and analysis by the Annoroad Gene Technology Company. Quantitative real-time PCR was performed for hepatocyte markers (p2, tfa, and itr) and proinflammatory cytokines (ccl20, il1b, il6, il18, mmp9, mpx, tnfa, and tnfb) using Fast-Start Universal SYBR Green Master Mix (Roche) following the manufacturer’s protocols, and their levels were normalized to that of β-actin. The primers used for these analyses are listed in Table 1.

E. coli–induced inflammation assays

The E. coli–induced inflammatory response assay was performed as previously described (35).

Quantification and statistical analysis

The liver volume of confocal Z-stack images was measured by Imaris version 9.0.2 for Windows. All statistical tests were performed with GraphPad Prism version 7.0 for Windows (GraphPad Software). The data were analyzed with Student’s t test, and multiple comparisons performed with analysis of variance tests were used to determine statistical significance. Statistical significance was defined as p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***).

Acknowledgments—We thank Dr. Li Li for providing us the Tg(mpeg1:EGFP) transgenic line and the JSH-23 inhibitor. Dr. Huang Honghui for providing Tg(cdh17:EGFP), and Dr. Wensheng Wei for kindly providing the pLentilox3.7 shRNA GFP vector plasmids.

References

1. Kawamoto, S., Matsumoto, Y., Mizuno, K., Oktubo, K., and Matsubara, K. (1996) Expression profiles of active genes in human and mouse livers. Gene 174, 151–158 CrossRef Medline
2. Pack, M., Solnicz-Krezel, L., Malicki, J., Neuhauss, S. C., Schier, A. F., Stemple, D. L., Driever, W., and Fishman, M. C. (1996) Mutations affecting development of zebrafish digestive organs. Development 123, 321–328 Medline
3. Field, H. A., Ober, E. A., Roeser, T., and Stainier D. Y. (2003) Formation of the digestive system in zebrafish: I. liver morphogenesis. Dev. Biol. 253, 279–290 CrossRef Medline
4. Janeway, C. A. (1989) Approaching the asymptote: evolution and revolution in immunology. Cold Spring Harbor Symp. Quant. Biol. 54, 1–13 CrossRef
5. Matzinger, P. (1994) Tolerance, danger, and the extended family. Annu. Rev. Immunol. 12, 991–1045 CrossRef Medline
6. Kubes, P., and Mehal, W. Z. (2012) Sterile inflammation in the liver. Gastroenterology 143, 1158–1172 CrossRef Medline

one-cell stage WT embryos as previously described (50). Acceptable adult founders were isolated and propagated. The plasmid carrying heat shock promoter-driven hsp70l:HA-zc3h8-H353Q-T2A-mCherry was mutated similarly to the cq5 mutant via site-directed mutagenesis as previously described (51).

Whole-mount in situ hybridization, fluorescent in situ hybridization, and antibody staining

Whole-mount in situ hybridization and fluorescent in situ hybridization were performed as previously described (12). Probes for the insulin, fabp2, fabp10a, mpx, il1b, nfkb1a, and zc3h8 transcripts were generated from linearized plasmids (insulin, fabp2, fabp10a, trypsin, and il1b) and PCR products (nfkb1a, nfkb1b, and zc3h8) using the digoxigenin RNA labeling kit (Roche Applied Science). Primers for trypsin (52) were designed based on the available data. The embryos were imaged using a Zeiss microscope (SteREO DiscoveryV20) equipped with Axio Vision Rel 4.8.2 software. Antibody staining was performed as previously described (10) using the following antibodies: anti-Dendra2 (1:100; Evrogen, Moscow, Russia), anti-GFP (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), S276-p65 (1:100; Abcam, Cambridge, MA), 2F11 (1:1000; Abcam, Cambridge, MA), and mCherry (1:100; Abcam, Cambridge, MA). ZEN2010 software equipped on an LSM780 confocal microscope (Carl Zeiss) was used for the imaging of antibody-stained larvae.

EdU staining and TUNEL assays

The EdU and TUNEL assays were performed as previously described (10).

Western blotting assays

Western blotting assays were performed as previously described (12). The hsp70l:HA-zc3h8-T2A-mCherry and hsp70l:HA-zc3h8H353Q-T2A-mCherry plasmids were injected into WT embryos, and the mCherry-positive cells detectable at 54 hpf were sorted by FACS for Western blotting analysis. Quantification of Western blotting densitometry band was analyzed by ImageJ software.

Heat shock and chemical inhibitor treatment

For the induction of Zc3h8 overexpression from Tg(hsp70l:HA-zc3h8-T2A-mCherry)cq43 embryos were placed in culture medium and then transferred to a 38.5 °C water bath for 35 min at the indicated stage. Larvae at 4 dpf were incubated with 15 mg/liter dexamethasone (Sangon Biotech, Shanghai, China) in the egg water for 2 consecutive days, and dexamethasone supplementation was performed every 24 h. Control larvae were incubated in egg water containing methanol (same volume as Dex). Larvae at 4 dpf were incubated with JSH-23 (5 µM; Selleck Chemicals, Houston, TX) in the egg water from 4 to 6 dpf, with the water being changed every 24 h. Control larvae were incubated in egg water containing DMSO (Sangon Biotech, Shanghai, China) (same volume as JSH-23).
Roles of Zc3h8 in digestive organ degeneration

7. Gao, B., Seki, E., Brenner, D. A., Friedman, S., Cohen, J. I., Nagy, L., Szabo, G., and Zakhari, S. (2011) Innate immunity in alcoholic liver disease. *Am. J. Physiol. Gastroint. Liver Physiol.* **300**, G516–G525 CrossRef Medline

8. Schroder, K., and Tschopp, J. (2010) The inflammasomes. *Cell* **140**, 821–832 CrossRef Medline

9. O’Neill, L. A. (2006) Targeting signal transduction as a strategy to treat inflammatory diseases. *Nat. Rev. Drug Discov.* **5**, 549–563 CrossRef Medline

10. Qi, F., Song, J., Yang, H., Gao, W., Liu, N. A., Zhang, B., and Lin, S. (2010) Systematic analysis of key regulators in liver regeneration (al) promotes liver outgrowth during zebrafish hepatogenesis. *PlOS One* **7**, e30835 CrossRef Medline

11. Thomas, I. A., Pope, C., Wojtach, D., Robson, A. J., Gordon-Walker, T. T., Hartland, S., Ramachandran, P., Van Deemter, M., Hume, D. A., Iredale, J. P., and Forbes, S. J. (2011) Macrophage therapy for murine liver fibrosis recruits host effector cells improving fibrosis, regeneration, and function. *Hepatology* **53**, 2003–2015 CrossRef Medline

12. Sunami, Y., Leithäuser, F., Gul, S., Fiedler, K., Guldiken, N., Espenalb, S., Holzmann, K. H., Hipp, N., Sindirilaru, A., Luedde, T., Baumann, B., Wissel, S., Krepfl, F., Schneider, M., Schräßfetter-Kochanek, K., et al. (2012) Hepatic activation of IKK/NF-κB signaling induces liver fibrosis via macrophage-mediated chronic inflammation. *Hepatology* **56**, 1117–1128 CrossRef Medline

13. Shen, H., Sheng, L., Chen, Z., Jiang, L., Su, H., Yin, L., Omary, M. B., and Rui, L. (2014) Mouse hepatocyte overexpression of NF-κB-inducing kinase (NIK) triggers fatal macrophage-dependent liver injury and fibrosis. *Hepatology* **60**, 2065–2076 CrossRef Medline

14. Barnes, P. J., and Karin, M. (1997) Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* **336**, 1066–1071 CrossRef Medline

15. Carballo, E., and Blackshear, P. J. (2001) Roles of tumor necrosis factor

16. Carballo, E., Lai, W. S., and Blackshear, P. J. (1998) Feedback inhibition of macrophage tumor necrosis factor-α production by tristetraprolin. *Science* **280**, 1001–1005 CrossRef Medline

17. Taylor, G. A., Carballo, E., Lee, D. M., Lai, W. S., Patel, D. D., Schenken, D. L., Gilkeson, G. S., Broxmeyer, H. E., Haynes, B. F., and Blackshear, P. J. (1999) A pathogenetic role for TNF-α in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin deficiency. *Immunity* **4**, 445–454 CrossRef Medline

18. Carballo, E., Lai, W. S., and Blackshear, P. J. (1998) Feedback inhibition of macrophage tumor necrosis factor-α production by tristetraprolin. *Science* **280**, 1001–1005 CrossRef Medline

19. Lai, W. S., Carballo, E., Stewart, J. L., Pardi, S. R., and Blackshear, P. J. (1999) Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor α mRNA. *Mol. Cell. Biol.* **19**, 4311–4323 CrossRef Medline

20. Carballo, E., and Blackshear, P. J. (2001) Roles of tumor necrosis factor-α receptor subtypes in the pathogenesis of the tristetraprolin-deficiency syndrome. *Blood* **98**, 2389–2395 CrossRef Medline

21. Carrick, D. M., Lai, W. S., and Blackshear, P. J. (2004) The tandem CCCH zinc finger protein tristetraprolin and its relevance to cytokine mRNA turnover and arthritis. *Arthritis Res. Ther.* **6**, 248–264 CrossRef Medline

22. Vinuesa, C. G., Cook, M. C., Angelucci, C., Athanassopoulos, V., Rui, L., Hill, K. M. Y., Wu, D., Domschken, H., Whittle, B., Lambe, T., Roberts, I. S., Copley, R. R., Bell, J. I., Cornwall, R. J., and Goodnow, C. C. (2005) A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoreactivity. *Nature* **435**, 452–458 CrossRef Medline

23. Huang, P., Xiao, A., Zhou, M., Zhu, Z., Lin, S., and Zhang, B. (2011) Heritable gene targeting in zebrafish using customized TALENs. *Nat. Biotechnol.* **29**, 699–700 CrossRef Medline

24. Cox, A. G., and Goessling, W. (2015) The lure of zebrafish in liver research: regulation of hepatic growth in development and regeneration. *Curr. Opin. Genet. Dev.* **22**, 153–161 CrossRef Medline

25. Qi, F., Song, J., Yang, H., Gao, W., Liu, N. A., Zhang, B., and Lin, S. (2010) Mmp23b promotes liver development and hepatocyte proliferation through the tumor necrosis factor pathway in zebrafish. *Hepatology* **52**, 2158–2166 CrossRef Medline

26. Huang, H., Ruan, H., Aw, M. Y., Hussain, A., Guo, L., Gao, C., Qian, F., Leung, T., Song, H., Kimelman, D., Wen, Z., and Peng, J. (2008) Mypt1-mediated spatial positioning of Bmp2-producing cells is essential for liver organogenesis. *Development* **135**, 3209–3218 CrossRef Medline

27. Li, Y., Faroq, M., Sheng, D., Chandramouli, C., Lan, T., Mahajan, N. K., Kini, R. M., Hong, Y., Lisowsky, T., and Ge, R. (2012) Augmenter of liver regeneration (al) promotes liver outgrowth during zebrafish hepatogenesis. *PlOS One* **7**, e30835 CrossRef Medline

28. Inokuchi, S., Aoyama, T., Miura, K., Osterreicher, C. H., Kodama, Y., Mizoguchi, K., Akira, S., Brenner, D. A., and Seki, E. (2010) Disruption of TAK1 in hepatocytes causes hepatic injury, inflammation, fibrosis, and carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 844–849 CrossRef Medline

29. Liang, J., Wang, J., Azfer, A., Song, W., Tromp, G., Kolattukudy, P. E., and Fu, M. (2008) A novel CCCH-zinc finger protein family regulates proin-
Roles of Zc3h8 in digestive organ degeneration

In vivo genome editing using a high-efficiency TALEN system. Nature 491, 114–118

Grabber, C., Ioly, J. S., and Wittbrodt, J. (2004) Highly efficient zebrafish transgenesis mediated by the meganuclease I-SceI. Methods Cell Biol. 77, 381–401

Zheng, L., Baumann, U., and Reymond, J. L. (2004) An efficient one-step site-directed and site-saturation mutagenesis protocol. Nucleic Acids Res. 32, e115

Mudumana, S. P., Wan, H., Singh, M., Korzh, V., and Gong, Z. (2004) Expression analyses of zebrafish transferrin, ifabp, and elastaseB mRNAs as differentiation markers for the three major endodermal organs: Liver, intestine, and exocrine pancreas. Dev. Dyn. 230, 165–173

Inflammatory activation of macrophages. J. Biol. Chem. 283, 6337–6346

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., and Schilling, T. F. (1995) Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310

Doyle, E. L., Booher, N. J., Standage, D. S., Voytas, D. F., Brendel, V. P., Vandyk, J. K., and Bogdanove, A. J. (2012) TAL effector–nucleotide targeter (TALE-NT) 2.0: tools for TAL effector design and target prediction. Nucleic Acids Res. 40, W117–W122

Bedell, V. M., Wang, Y., Campbell, J. M., Poshusta, T. L., Starker, C. G., Krug, R. G., 2nd, Tan, W., Penheiter, S. G., Ma, A. C., Leung, A. Y., Fahrenkrug, S. C., Carlson, D. F., Voytas, D. F., Clark, K. J., Essner, J. J., et al. (2012) In vivo genome editing using a high-efficiency TALEN system. Nature 491, 114–118