Loss of the RNA-binding protein Rbm15 disrupts liver maturation in zebrafish

Liang Hu, Hongyan Li, Zhiping Chi, and Jianbo He*

From the Institute of Developmental Biology and Regenerative Medicine, Southwest University, Beibei, Chongqing, China

Received for publication, April 26, 2020, and in revised form, June 3, 2020 Published, Papers in Press, June 9, 2020, DOI 10.1074/jbc.RA120.014080

LBerry of the American Society for Biochemistry and Molecular Biology, Inc.

Liver organogenesis begins with hepatic precursors in the foregut endoderm, followed by hepatoblast specification, differentiation, outgrowth, and maturation for the formation of functional hepatocytes. Although several signaling pathways and critical factors that regulate liver specification, differentiation, and proliferation have been identified, little is known about how liver maturation is regulated. Here, we used a screen for mutations affecting liver development in zebrafish and identified a genetic mutant that exhibits a specific defect in liver maturation. Results from positional cloning revealed that Rbm15 is specifically expressed in the liver after its differentiation. CRISPR/Cas9-mediated loss of Rbm15 repressed hepatic maturation, but did not affect hepatoblast specification, differentiation, and hepatocyte proliferation and apoptosis. Additional experiments disclosed that the mTOR complex 1 (mTORC1) pathway is highly involved in RNA nuclear export (26). However, the role of Rbm15 in embryonic liver development has not been reported. We found that Rbm15 is an evolutionarily conserved Spen family protein and known to play a crucial role in RNA m6A modification, nuclear export, and alternative splicing. Rbm15 is an evolutionarily conserved Spen family protein and known to play a crucial role in RNA m6A modification, nuclear export, and alternative splicing. Rbm15 can affect promoter activity of Notch target genes, such as Hes1, in a cell-specific manner. It can repress Hes1 mRNA level in hematopoietic cell lines but enhance the Hes1 m6A modification in nonhematopoietic cells but enhance the Hes1 mRNA level in hematopoietic cells lines (22). Rbm15 can also affect hematopoietic stem cell and megakaryocyte development via regulating c-myc expression (23). m6A is the most widespread RNA modification; the establishment of m6A relies on RBM15 (24, 25). RBM15 is also involved in RNA nuclear export (26). However, the role of Rbm15 in liver development is unknown.
Zebrafish has been developed into a popular model organism in recent years because of advantages of their transparent embryos developing outside of the mother, allowing constant visualization, and 69% of all zebrafish genes have a clear human orthologue (27). High genetic conservation among vertebrates makes zebrafish an excellent model system to study liver development and disease (27, 28). Here we used chemical mutagenesis to screen genes involved in liver development and got a specific liver maturation mutant, \textit{cq96}. The mutant \textit{cq96} revealed normal liver specification, differentiation, and outgrowth, but the liver maturation was repressed. The development of the pancreas and intestine showed normal in \textit{cq96} mutant. Positional cloning and knockout experiments confirmed this mutation site located in gene \textit{rbm15}. Deficiency of Rbm15 leads to up-regulation of the activity of mTORC1 signaling, and inhibition of the mTORC1 pathway by rapamycin can rescue liver maturation defect. Furthermore, the treatment of rapamycin enhanced the nuclear import of Hnf4a and restored hepatic gene expression. Our data revealed an unexpected function of \textit{rbm15} in liver development in zebrafish.

Results

The \textit{cq96} mutant confers a liver maturation defect

We have screened mutagenized zebrafish larvae using transgenic line \textit{Tg(lfabp:Dendra2-NTR)} (29) and identified the \textit{cq96} mutant. At 5 dpf, this mutant showed normal body morphology but weaker Dendra2 expression (Fig. 1A). The confocal image exhibited relatively normal liver size but heterogeneous Dendra2 expression in the mutant (Fig. 1B). The expressions of digestive organ–specific markers \textit{trypsin} (exocrine pancreas) and \textit{ifabp} (intestine) were normal, but the hepatocyte-specific gene \textit{cp} was repressed (Fig. 1C). The expressions of functional markers of hepatocytes such as \textit{uox}, \textit{gc}, and \textit{gsy2} were repressed in the mutant (Fig. 1D and Fig. S1 (A and B)). These data indicate that the \textit{cq96} mutant specifically affects liver development and especially regulates liver maturation.

Zebrafish \textit{cq96} mutation site locates in gene \textit{rbm15}

To determine the target gene of the \textit{cq96} mutant, we performed genome mapping and placed the \textit{cq96} mutation site locus to gene \textit{rbm15}. The genomic sequencing result showed that there are 347 bp deleted in \textit{rbm15} exon 1, leading to translation into a truncated peptide (Fig. 2, A and B). To further confirm that \textit{rbm15} affects liver maturation, we generated a new \textit{rbm15} mutant by CRISPR/Cas9 that resembled the \textit{cq96} phenotype (Fig. 2, A–F). These results revealed that the mutation gene of \textit{cq96} is \textit{rbm15}. Furthermore, \textit{rbm15} expressed in the liver region from 4 dpf and much stronger at 5 dpf (Fig. 2D). The weak expressions of Dendra2 and hepatic maturation markers such as \textit{uox} and \textit{cp} in \textit{cq96} mutant can be rescued by using \textit{Rbm15} overexpression line \textit{Tg(hsp70l:rbm15-Flag\textsuperscript{cq97})} (Fig. 2, E–G). These results suggested that the mutation gene in \textit{cq96} is \textit{rbm15}.

Zebrafish \textit{cq96} mutant affects liver maturation but not hepatoblast specification

To evaluate hepatoblast specification in the \textit{rbm15} mutant, we assayed the expression of transcriptional factors important for hepatoblast formation and specification at 60 hpf. Interestingly, the expressions of \textit{prox1}, \textit{gata6}, \textit{hhex}, and \textit{foxa3} were not greatly different between mutants and siblings (Fig. 3A). This means that liver specification was normal in the \textit{cq96} mutant. Then we performed antibody staining for hepatic factors Hnf4a and Prox1 at 5 dpf. The nuclear signal for Hnf4a and Prox1 was much weaker compared with siblings (Fig. 3, B–E). These results suggest that the \textit{cq96} mutant has liver maturation rather than liver specification defects.

Loss of \textit{Rbm15} confers normal hepatic proliferation and apoptosis

To investigate the underlying mechanism of liver development defect in \textit{cq96} mutant, we performed antibody staining for PCNA, which labeled cells outside of the G\textsubscript{0} phase. We detected a similar ratio of PCNA and Dendra2 double-positive cells in siblings and mutants (Fig. 4, A and B). To assess whether cell death contributes to liver development defect in \textit{cq96} mutant, we performed a transferase-mediated dUTP nick-end labeling (TUNEL) assay on WT and \textit{cq96} at 5 dpf. Larval hepatocytes presented a low apoptotic index at 5 dpf, which was unchanged in \textit{cq96} mutant (Fig. 4C). Therefore, liver development defect in the \textit{cq96} mutant does not appear to be due to defective cell proliferation and apoptosis.

Inhibiting mTORC1 signaling pathway partially rescues the phenotype of \textit{cq96} mutant

Hyper- and hypoactivated mTORC1 pathway will impair normal liver development (30, 31). To explore whether the loss of \textit{rbm15} will affect the mTORC1 signaling or not, we performed antibody staining to check the expression level of p-4Ebp1, which indicates the activity of mTORC1 signaling, and found that the \textit{rbm15}\textsuperscript{+/--} mutant larvae showed high p-4Ebp1 level in the liver (Fig. 5A). This indicates that the mTORC1 pathway was hyperactivated in the \textit{rbm15}\textsuperscript{-/-} liver. To further confirm that the liver maturation defect in the \textit{rbm15} mutant was caused by mTORC1 activation, we used 10 \textmu M rapamycin to inhibit mTORC1 from 5 to 7 dpf. mTORC1 inhibition can partially rescue the developmental liver defect (Fig. 5 (B and C) and Fig. S2A). After rapamycin treatment, the protein level of Hnf4a in mutant hepatocyte was rescued (Fig. 5, D and E), but the mRNA level of \textit{hnf4a} showed no big difference between WT, \textit{rbm15}\textsuperscript{-/-}, and sample groups (Fig. S2B). Furthermore, the expressions of hepatocyte-specific genes such as \textit{cp} and \textit{gc} were also restored after rapamycin treatment (Fig. 5F). These results indicate that inhibition of mTORC1 can partially rescue liver maturation defects in \textit{rbm15} mutant.

Discussion

We have described a liver developmental mutant caused by \textit{rbm15} mutation, and inhibition of mTORC1 was an efficient strategy to rescue hepatic maturation defects. We showed that

\textbf{Rbm15 regulates liver maturation}

\textbf{Zebrafish cq96 mutant affects liver maturation but not hepatoblast specification}

\textbf{Loss of Rbm15 confers normal hepatic proliferation and apoptosis}

\textbf{Inhibiting mTORC1 signaling pathway partially rescues the phenotype of cq96 mutant}

\textbf{Discussion}
loss of Rbm15 specifically affected hepatic maturation, but not the developmental progress of intestine, pancreas. The mutant liver exhibited relative normal proliferation and apoptosis but weak hepatic gene expression. The two important hepatic transcriptional factors Hnf4a and Prox1 exhibited weak nuclear location, which could explain why rbm15 mutant showed hepatic maturation defects to some degree. Liver failure caused by rbm15 mutation also showed abnormal mTORC1 activation. Inhibition of mTORC1 can partially recover hepatic gene expression; this progress may rely on the enhancement of Hnf4a nuclear import.

Rbm15 is an important post-transcriptional regulator involved in RNA nuclear export, m6A modification, and alternative splicing (21, 25, 26). It is indispensable for megakaryocyte differentiation, and rbm15 defect can induce acute megakaryoblast leukemia (20). mTORC1 acts as a metabolic regulator important for cell growth and differentiation (19). Abnormal mTORC1 activation is associated with liver developmental defects and enhancement of liver damage (30, 32). Our finding first reveals that rbm15 is essential for hepatic maturation and that loss of rbm15-induced liver developmental failure partially depends on aberrant high mTORC1 activation.

We point out the importance of rbm15 in hepatic maturation, but we still do not know the target genes of rbm15. The relationship between rbm15 deficiency and high mTORC1 activity is still a mystery. RNA immunoprecipitation sequencing experiments will be indispensable to further answer these questions.

Experimental procedures

Ethics statement

All experimental protocols were approved by the Institute of Developmental Biology and Regenerative Medicine, Southwest University (Chongqing, China), and the methods 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 of 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 from Southwest University (2007).

Zebrafish lines

Zebrafish (Danio rerio) AB strain-derived Tg(lfabp:Dendra2-NTR)cq1 was used as WT, and rbm15cq96 mutant was generated by ENU treatment. The IND line was used for mapping. These zebrafish lines were raised under standard conditions, and embryos/larvae for the experiment were treated with 0.003% PTU (Sigma) from 24 hpf.

CRISPR/Cas9-targeted rbm15 knockout

The CRISPR/Cas9 was carried out essentially as reported previously (33). The sequence for CRISPR RNA is shown in Fig. 2A. We used the following primers to identify the genotype of
mutant: forward primer, 5' -GAATTCTGGCGGAGGAAGCA-3'; reverse primer, 5' -AAGCCGACCCAGTGCTAAC-3'.

**Whole-mount in situ hybridization and fluorescent in situ hybridization**

Whole-mount *in situ* hybridization and fluorescent *in situ* hybridization were based on a previous report (29) using anti-sense probes for *hhex*, *gata6*, *foxa3*, *prox1*, *uox*, *gc*, *cp*, *hnf4a*, and *rbm15*. Primers used for amplifying the *rbm15* probe were 5' -GAGGCAGTTTACTTGAACAG-3' (forward primer) and 5' -AAGCCGACCCAGTGCTAAC-3' (reverse primer).

**Antibody staining and TUNEL assay**

Antibody staining and TUNEL assay were performed as described previously (29). The following antibodies were used: antibodies against Dendra2 (1:1000; AB821, Evrogen, Moscow, Russia), phospho-4E-BP1 (Thr-37/46) (1:500; catalog no. 2855, Cell Signaling), Hnf4a (1:200; sc-6556, Santa Cruz Biotechnology, Inc.), Proxl (1:500; ab5475, Chemicon), 2F11 (1:1000; ab71826, Abcam, Cambridge, MA), and PCNA (1:1000; SAB2701819, Sigma).

**Generation of transgenic line for rescue experiments**

Full-length *rbm15* cDNA was amplified by PrimeSTAR HS DNA Polymerase (Takara) and cloned into pBluescript vector. The full-length *rbm15*-Flag CDS was driven by hsp70l promoter, and the plasmid was injected into AB strain embryos to generate the transgenic line Tg(hsp70l:rbm15-Flag)cq97. Positive embryos showed cerulean expression in the eyes of the offspring.

**Rapamycin treatment and heat shock**

Embryos were treated with 10 μM rapamycin (Sangon Biotech, Shanghai, China) in PTU egg water from 5 to 7 dpf and replaced rapamycin solution every 24 h. The control group was treated with

---

**Figure 2. The mutation gene in cq96 is *rbm15***

A and B, schematic drawing of the *rbm15* locus, *cq96*, CRISPR/Cas9 targeting site (green), and the PAM sequence (red); C, genomic sequencing result of *rbm15* mutation derived from the CRISPR/Cas9 knockout experiment. D, WISH and fluorescence *in situ* hybridization (FISH) results showing the expression pattern of *rbm15* from 24 hpf to 5 dpf. E, heat shock transgenic line Tg(hsp70l:rbm15-Flag)cq97 rescues liver defects of *cq96*. F, evaluating the rescue effects by detecting the expression of liver functional genes *uox* and *gc* via WISH. Numbers indicate the proportion of larvae exhibiting the expression shown. Asterisks indicate statistical significance: ****, p < 0.0001. Scale bars, 100 μm; error bars, S.D.
0.2% DMSO. To induce rbm15 overexpression from Tg(hsp70l:rbm15-Flag)q97, larvae were placed in egg water and then incubated in a 38.5°C water bath for 30 min once a day from 5 to 7 dpf.

**Data collection and analysis**
All images were taken on a SteREO DiscoveryV20 microscope (Carl Zeiss, Germany) and LSM880 confocal microscope.
The intensities of fluorescent images were measured with ImageJ. The statistical analysis were performed with GraphPad Prism 8. Variation of individual data points was represented in S.D.

Data availability

All the data are contained within the article.

Acknowledgments—We are grateful to Prof. Lingfei Luo for guidance and assistance; Jinzi Chen and Kai Gang for discussions; and Qifen Yang, Rui Ni, and Xuemei Tang for technical assistance.

Author contributions—L. H. and J. H. conceptualization; L. H. and H. L. data curation; L. H. and H. L. formal analysis; L. H., H. L., and Z. C. methodology; L. H. writing-original draft; J. H. funding acquisition; J. H. writing-review and editing.

Funding and additional information—This work was supported by National Key R&D Program of China Grant 2019YFA0802703 (to J. H.) and National Natural Science Foundation of China Grants 31970784 and 31801214 (to J. H.).

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: hpf, hours post fertilization; dpf, days post fertilization; PTU, 1-phenyl 2-thiourea; WISH, whole-mount in situ hybridization; mTOR, mechanistic target of rapamycin; PCNA, proliferating cell nuclear antigen; TUNEL, transferase-mediated dUTP nick-end labeling.

References

1. Berasain, C., and Avila, M. A. (2015) Regulation of hepatocyte identity and quiescence. Cell. Mol. Life Sci. 72, 3831–3851 CrossRef Medline
2. Gordillo, M., Evans, T., and Gouon-Evans, V. (2015) Orchestrating liver development. Development 142, 2094–2108 CrossRef Medline
3. Field, H. A., Ober, E. A., Roesser, T., and Stainier, D. Y. (2003) Formation of the digestive system in zebrafish. I. Liver morphogenesis. Dev. Biol. 253, 279–290 CrossRef Medline
4. Calmont, A., Wandzioch, E., Tremblay, K. D., Minowada, G., Kaestner, K. H., Martin, G. R., and Zaret, K. S. (2006) An FGF response pathway that mediates hepatic gene induction in embryonic endoderm cells. Dev. Cell 11, 339–348 CrossRef Medline
5. Shin, D., Shin, C. H., Tucker, J., Ober, E. A., Rentzsch, F., Poss, K. D., Hammerschmidt, M., Mullins, M. C., and Stainier, D. Y. (2007) Bmp and Fgf signaling are essential for liver specification in zebrafish. Development 134, 2041–2050 CrossRef Medline

Figure 5. Inhibiting the mTORC1 pathway partially rescues the liver maturation defects in rbm15<sup>cq96</sup>. A, confocal images (3D) showing the antibody staining of p-4Ebp1 and 2F11 to evaluate the activity of mTORC1 signal in hepatocytes at 5 dpf. B, rapamycin treatment and confocal images reflecting the rescue efficiency of rapamycin treatment. C, quantification of Dendra2 fluorescent intensity in WT, cq96, and rapamycin treatment. D, antibody staining results of Hnf4a and Dendra2 showing that rapamycin treatment affects the cellular and protein levels of Dndera2 and Hnf4a. E, the quantification of Hnf4a fluorescent intensity in WT, cq96, and rapamycin treatment. F, WISH images showing the expression patterns of <i>cp</i> and <i>gc</i> after rapamycin treatment. <i>ropa</i>, rapamycin. The numbers indicate the proportion of larvae exhibiting the expression shown. Asterisks indicate statistical significance: ****, <i>p</i> < 0.0001. Scale bars, 100 μm; error bars, S.D.
Rbm15 regulates liver maturation

6. Ober, E. A., Verkade, H., Field, H. A., and Stainier, D. Y. R. (2006) Meso-
dermal Wnt2b signalling positively regulates liver specification. Nature 442, 688–691. CrossRef Medline

7. Watanabe, H., Takayama, K., Inamura, M., Tachibana, M., Mimura, N., Katayama, K., Tashiro, K., Nagamoto, Y., Sakurai, F., Kawabata, K., Furue, M. K., and Mizuguchi, H. (2014) HHEX promotes hepatic-lineage specification through the negative regulation of eomesodermin. PLoS One 9, e9079. CrossRef Medline

8. Lee, C. S., Friedman, J. R., Fulmer, J. T., and Kaestner, K. H. (2005) The ini-
tiation of liver development is dependent on Foxa transcription factors. Gastroenterology 131, 1561–1572. CrossRef Medline

9. Zhao, R., Watt, A. J., Li, J., Luebke-Wheeler, J., Morrisey, E. E., and Dun-
scan, S. A. (2005) GATA6 is essential for embryonic development of the liver but dispensable for early heart formation. Mol. Cell Biol. 25, 2622–2631. CrossRef Medline

10. Tan, X., Behari, J., Cieply, B., Michalopoulos, G. K., and Monga, S. P. (2006) Conditional deletion of β-catenin reveals its role in liver growth and regeneration. Gastroenterology 131, 1561–1572. CrossRef Medline

11. Colletti, M., Cicchini, C., Conigliaro, A., Santangelo, L., Alonzi, T., Pas-
quini, E., Tripodi, M., and Amicone, L. (2009) Convergence of Wnt signaling on the HNF4α-driven transcription in controlling liver zonation. Gastroenterology 137, 660–672. CrossRef Medline

12. Yilmalı, D., Christodoulou, C., Galli, G. G., Yang, K., Pepe-Mooney, B., Gurung, B., Shrestha, K., Cahan, P., Stanger, B. Z., and Camargo, F. D. (2014) Hippo pathway activity influences liver cell fate. Cell 157, 1324–1338. CrossRef Medline

13. Zong, Y., Panikkar, A., Xu, J., Antoniou, A., Raynaud, P., Lemaigne, F., and Stanger, B. Z. (2009) Notch signaling controls liver development by regulating biliary differentiation. Development 136, 1727–1739. CrossRef Medline

14. Chaturantabut, S., Shwartz, A., Garnaas, M. K., LaBella, K., Li, C. C., Car-
roll, K. J., Cutting, C. C., Budrow, N., Palaria, A., Gorelick, D. A., Tremblay, K. D., North, T. E., and Goessling, W. (2020) Estrogen acts via estrogen receptor 2b to regulate hepatobiliary fate during vertebrate development. Hepatology CrossRef Medline

15. Lüdtke, T. H., Christoffels, V. M., Petry, M., and Kispert, A. (2009) Tbx3 promotes liver bud expansion during mouse development by suppression of cholangiocyte differentiation. Hepatology 49, 969–978. CrossRef Medline

16. Hang, H.-L., Liu, X.-Y., Wang, H.-T., Xu, N., Bian, J.-M., Zhang, J.-J., Xia, Q., and Xia, Q. (2017) Estrogen activation of G-protein-coupled estrogen receptor 1 regu-
lates phosphoinositide 3-kinase and mTOR signaling to promote liver growth in zebrafish and proliferation of human hepatocytes. Gastroenterology 156, 1788–1804.e13. CrossRef Medline

17. Zhao, R., Watt, A. J., Li, J., Luebke-Wheeler, J., Morrisey, E. E., and Dun-
scan, S. A. (2005) GATA6 is essential for embryonic development of the liver but dispensable for early heart formation. Mol. Cell Biol. 25, 2622–2631. CrossRef Medline

18. Chaturantabut, S., Shwartz, A., Evasion, K. J., Cox, A. G., Labela, K., Schepers, A. G., Yang, S., Acuna, M., Houyras, Y., Mancio-Silva, L., Romano, S., Gorelick, D. A., Cohen, D. E., Zon, L. I., Bhatia, S. N., et al. (2019) Estrogen activation of G-protein-coupled estrogen receptor 1 regu-
lates phosphoinositide 3-kinase and mTOR signaling to promote liver growth in zebrafish and proliferation of human hepatocytes. Gastroenterology 156, 1788–1804.e13. CrossRef Medline

19. Tran, N. T., Su, H., Khodadadi-Jamayran, A., Lin, S., Zhang, L., Zhou, D., Pawlik, K. M., Townes, T. M., Chen, Y., Mulloy, J. C., and Zhao, X. (2016) The AS-RBM15 lncRNA enhances RBM15 protein translation during megakaryocyte differentiation. EMBO Rep. 17, 887–900. CrossRef Medline

20. Zhang, L., Tran, N. T., Su, H., Wang, R., Lu, Y., Tang, H., Aoyagi, S., Guo, A., Khodadadi-Jamayran, A., Zhou, D., Qian, K., Hricik, T., Cote, J., Han, X., Zhou, W., et al. (2015) Cross-talk between PRMT1-mediated methyla-
tion and ubiquitylation on RBM15 controls RNA splicing. eLife 4, e07938. CrossRef Medline

21. Liu, C., Zhang, J., Breslin, P., Onciu, M., Ma, Z., and Morris, S. W. (2009) c-Myc is a target of RNA-binding motif protein 15 in the regulation of adult hematopoietic stem cell and megakaryocyte development. Blood 114, 2087–2096. CrossRef Medline

22. Nakayama, K., Tashiro, K., Nagamoto, Y., Sakurai, F., Kawabata, K., Furue, M. K., and Mizuguchi, H. (2014) HHEX promotes hepatic-lineage specification during liver disease research. Hepatology 59, 1361–1377. CrossRef Medline

23. Chi, J., and Sadler, K. C. (2009) New school in liver development: lessons from zebrafish. Hepatology 50, 1656–1663. CrossRef Medline

24. He, J., Lu, H., Zou, Q., and Luo, L. (2014) Regeneration of liver after extreme hepatocyte loss occurs mainly via biliary transdifferentiation in zebrafish. Gastroenterology 146, 789–800.e8. CrossRef Medline

25. He, J., Yang, Y., Zhang, J., Chen, J., Wei, X., He, J., and Luo, L. (2017) Ribo-
mosome biogenesis protein Urbi1 acts downstream of mTOR complex 1 to modulate digestive organ development in zebrafish. J. Genet. Genomics 44, 567–576. CrossRef Medline

26. Zhu, Q., Wang, H., Jiang, B., Ni, X., Jiang, L., Li, C., Wang, X., Zhang, F., Ke, B., and Lu, L. (2018) Loss of ATGF3 exacerbates liver damage through the activation of mTOR/p70S6K/HIF-1α signaling pathway in liver inflammatory injury. Cell Death Dis. 9, 910. CrossRef Medline

27. Chang, N., Sun, C., Gao, L., Zhu, D., Xu, X., Zhu, X., Xiong, J. W., and Xi, J. J. (2013) Genome editing with RNA-guided Cas9 nuclease in zebrafish embryos. Cell Res. 23, 465–472. CrossRef Medline