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Validation of Protein Knockout in Mutant Zebrafish Lines Using *In Vitro* Translation Assays

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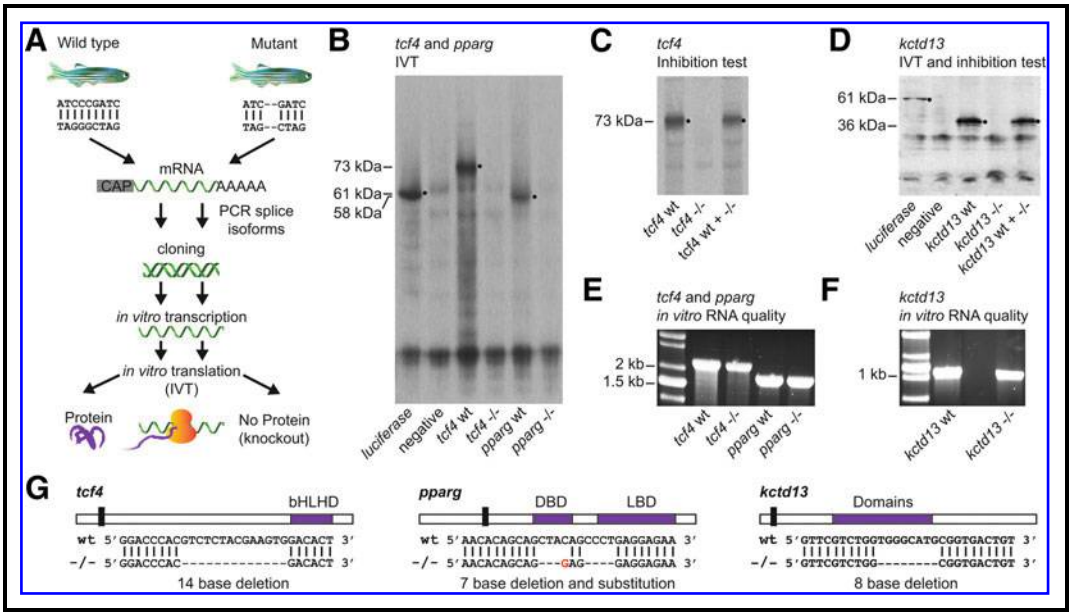

FIG. 1. *In vitro* translation (IVT) assay to verify protein loss-of-function in mutants. **(A)** Schematic of procedure. cDNA is produced from mRNA derived from wild type (wt) or mutant fish, cloned, transcribed *in vitro* and translated *in vitro* to test for knockout. **(B)** IVT outcomes. Proteins are detected by chemiluminescence (Supplementary Methods). Only wt RNAs produce protein in IVT reactions, demonstrating mutants are likely to be knockouts. Expected bands of 73 kDa (*dot*) and 58 kDa (*dot*) are detected for *tcf4* wt and *pparg* wt, respectively, demonstrating that mutants are effective knockouts. Luciferase: positive control (61 kDa, *dot*); negative: no RNA. **(C)** Trans-inhibition control. To determine whether the *tcf4* mutant RNA nonspecifically inhibits translation, a mixing control is performed. Expected bands of 73 kDa (*dots*) are obtained with wt and a 1:1 mixture of wt and mutant mRNA. **(D)** IVT outcomes. Only wt RNAs produce protein in IVT reactions, demonstrating mutants are likely to be knockouts. Thirty-six kilodalton proteins corresponding to Kctd13 (*dots*) are obtained with wt and equimolar wt:mutant mix. Luciferase: positive control (61 kDa, *dot*); negative: no RNA. **(E)** *In vitro* transcribed RNA quality control. RNA is observed after ethidium bromide staining. Single bands of 2 and 1.5 kb are detected for wt and mutant *tcf4* and *pparg* mRNAs, respectively. No degradation is observed. **(F)** *In vitro* transcribed RNA quality control. RNA was observed after ethidium bromide staining. A single band of 1 kb is detected at equivalent sizes for wt and mutant *kctd13*. No degradation is observed. **(G)** Sequences and schematic representation of mutated genes and proteins. bHLHD, basic helix-loop-helix domain; *Black box*, predicted location of truncation; DBD, DNA binding domain; LBD, ligand binding domain.

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Abstract

Advances in genome-editing technology have made creation of zebrafish mutant lines accessible to the community. Experimental validation of protein knockout is a critical step in verifying null mutants, but this can be a difficult task. Absence of protein can be confirmed by Western blotting; however, this approach requires target-specific antibodies that are generally not available for zebrafish proteins. We address this issue using *in vitro* translation assays, a fast and standard procedure that can be easily implemented.

Main text

A CHALLENGE IN CHARACTERIZING zebrafish mutants is how to determine whether the mutated gene produces protein. When a forward genetic screen is performed, DNA changes found in a mutant clearly change gene function since mutant identification is based on phenotype. However, when genome-editing methods or TILLING[1] is used to create mutations,[2–5] assessing whether gene function has been affected can be difficult, especially if no obvious phenotype is present. DNA derived from targeted genes is generally analyzed computationally, and mutant lines are chosen where insertion, deletion, or point mutations predict premature stop codons and a truncated protein. However, mutations may alter splicing leading to new splice isoforms that may be translated,[6] alternative translation start sites may be used,[7,8] or internal reinitiation of protein synthesis may occur.[9] Experimental validation of protein traditionally relies on Western blot or immunostaining; however, these require a target-specific antibody and most zebrafish proteins do not have a corresponding antibody available.

To meet this challenge, we have used *in vitro* translation (IVT)[10] assays to assess whether genes mutated by gene editing are able to produce protein. To test whether a zebrafish mutant RNA is translated (Fig. 1A), we used the following steps (Supplementary Methods and Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/zeb). (1) RNA is isolated from homozygous mutant and wild type fish at a time when the gene is expressed (e.g. 48 hpf larvae, 24 hpf embryos, and adult fin clips were used for *tcf4*, *kctd13*, and *pparg* RNA exactions, respectively); (2) cDNA is produced by reverse transcription; (3) the target gene is amplified using PCR; *tcf4*, *kctd13*, and *pparg* have a single isoform reported,[11,12] data that were corroborated by RNAseq data of splice wild type isoform analysis; (4) PCR products are cloned into an RNA expression vector; (5) RNA is produced from each clone by *in vitro* transcription; (6) protein is produced from each RNA by IVT using commercial extracts; and (7) resulting proteins are resolved by SDS-PAGE and detected. We used rabbit reticulocyte lysate coupled with Transcend™ tRNA (Promega) to biotin label the proteins and chemiluminescent detection through membrane blotting using streptavidin-HRP. However, other methods can be used to detect proteins, including autoradiography or fluorescence. The protocol can be completed in ~1–2 weeks. We have applied these methods to validate the *tcf4*$^{-/-}$, *pparg*$^{-/-}$, and *kcdt13*$^{-/-}$ mutant lines generated by TALEN[2] or CRISPR/Cas 9 technologies[3–5] (Fig. 1B–D, G). Controls include monitoring RNA quality (Fig. 1E, F) and testing *in vitro* RNA for trans-inhibition of IVT using a mixing assay (Fig. 1C, D).

Certain considerations or limitations of this methodology apply. First, the method is simplest when the homozygous mutant is viable (for some period when the gene of interest is expressed) to isolate a pure loss-of-function RNA population. RNA from heterozygotes can be used if cDNA clones corresponding to the mutant are characterized. Second, transmembrane or membrane-associated proteins may not be readily translated in reticulocyte lysates or may aggregate. Adding canine pancreatic microsomal membranes or the detergent octaethylene glycol mono n-dodecyl ether (Nikkol)[13] may reduce aggregation and promote translation. Another alternative for membrane-associated proteins may be 1-Step Coupled Human IVT Kits (Thermo Fisher). Third, if it is known that a gene can have multiple mRNA splice isoforms, appropriate PCR primers should be used to identify these to test protein production from all isoforms that may be targets of the mutation or change with mutation.

However, identifying splice variants or testing whether anomalous new splice products occur after mutation is not a trivial task. Databases may lack the information needed and computational methods are not fully reliable. Empirical methods such as 5′ or 3′ RACE, or splice assembly from RNAseq data of wild type and mutants may be necessary. It is also possible that internal translation reinitiation may occur, producing small proteins with some function, and appropriate gel conditions are required to detect these. Fourth, recent studies have shown that core ribosomal proteins, rRNAs, and 5′UTR cis-regulatory elements can act as critical regulators of ribosome activity, playing an important role in cell- and tissue-specific regulation of mRNA translation.[14] Thus, the reticulocyte lysate system may not completely mimic the translational conditions present in the embryo. Despite these considerations, using IVT approaches can lend confidence to assessment of whether a mutant allele can produce protein.

In summary, IVT assays can readily be used to assess protein loss-of-function in RNA derived from mutant zebrafish lines. With increasing use of genome-editing approaches in zebrafish, this approach can help researchers determine whether mutant lines are likely to be loss-of-function.

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Author Contributions

B.S.C., C.C.C., and H.L.S. designed the experiments. B.S.C., C.C.C., and X.C. performed experiments. B.S.C., C.C.C., and J.M.M. provided reagents. B.S.C., C.C.C., J.M.M., and H.L.S. wrote the article; all authors contributed to editing the article.

Disclosure Statement

No competing financial interests exist.

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