

Generation of *ints14* Knockout Zebrafish using CRISPR/Cas9 for the Study of Development and Disease Mechanisms

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Conflict of interests

The authors declare no potential conflict
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Authors' contributions

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Abstract

INTS14/VWA9, a component of the integrator complex subunits, plays a pivotal role in regulating the fate of numerous nascent RNAs transcribed by RNA polymerase II, particularly in the biogenesis of small nuclear RNAs and enhancer RNAs. Despite its significance, a comprehensive mutation model for developmental research has been lacking. To address this gap, we aimed to investigate the expression patterns of *INTS14* during zebrafish embryonic development. We generated *ints14* mutant strains using the CRISPR/Cas9 system. We validated the gRNA activity by co-injecting Cas9 protein and a single guide RNA into fertilized zebrafish eggs, subsequently confirming the presence of a 6- or 9-bp deletion in the *ints14* gene. In addition, we examined the two mutant alleles through PCR analysis, T7E1 assay, TA-cloning, and sequencing. For the first time, we used the CRISPR/Cas9 system to create a model in which some sequences of the *ints14* gene were removed. This breakthrough opens new avenues for in-depth exploration of the role of *ints14* in animal diseases. The mutant strains generated in this study can provide a valuable resource for further investigations into the specific consequences of *ints14* gene deletion during zebrafish development. This research establishes a foundation for future studies exploring the molecular mechanisms underlying the functions of *ints14*, its interactions with other genes or proteins, and its broader implications for biological processes.

Keywords: *INTS14*, CRISPR/Cas9, Zebrafish, Mutation, RNA polymerase II

INTRODUCTION

The C-terminal domain (CTD) of RNA polymerase II (RNAPII) plays a crucial role in the transcriptional regulation and RNA processing of protein-coding genes. Additionally, substantial evidence suggests that the CTD is involved in the transcription and processing of spliceosomal U small nuclear RNAs (U1 to U5 snRNAs) dependent on RNAPII. The integrator complex (INT) is responsible for processing the 3'-end of spliceosomal snRNAs and controls the transcription of protein-coding genes by facilitating transcription termination following unstable pausing. In 2005, Baillat et al. made this discovery as part of their initiative to identify interacting partners of DSS1 (Deleted in split hand/split foot1), a protein linked to congenital limb defects in humans (Baillat et al., 2005). INT is

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Ethics approval

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absent in lower eukaryotes, such as yeast, but is conserved in higher eukaryotes (Sabath et al., 2020). It comprises at least 14 subunits in mammalian cells, and for the majority of these subunits, both their function and structure remain uncharacterized. (Welsh & Gardini, 2023).

After the discovery of the integrator, it has become increasingly apparent that this complex can regulate the processing and expression of RNA PolII transcripts beyond snRNAs. These transcripts include a range of nonpolyadenylated RNAs, such as enhancer RNA (eRNA), telomerase RNA, viral miRNAs, and replication-dependent histone, along with the transcription of numerous canonical protein-coding genes and long noncoding (lnc) RNAs (Baillat et al., 2005). The dysregulation of integrator expression has been associated with developmental and disease phenotypes in various metazoan species. This correlation is not unexpected, as mutation in the integrator can result in the dysregulation of the expression of hundreds to thousands of mRNAs. Regarding the role of IntS13 in human myeloid differentiation, an increasing body of research indicates that integrator subunits play an essential role in normal development across metazoans (Mendoza-Figueroa et al., 2020). The mutation in the integrator complex subunit 1 gene (*c*) and subunit 8 gene (*INTS8*) in humans have been demonstrated to induce a severe neurodevelopmental syndrome. This syndrome is characterized by profound intellectual disability, epilepsy, rigidity, facial and limb dysmorphisms as well as subtle structural abnormalities in the brain (Peart et al., 2013; Oegema et al., 2017; Krall et al., 2019). In addition, the deletion or mutation of integrator subunits in *Mus musculus* (*Ints1*), *Oryzias latipes* (*Ints1*), *Drosophila melanogaster* (*IntS4* and *7*), *Caenorhabditis elegans* (*Ints6*), and *Danio rerio* (*IntS6*) have all been reported to result in abnormalities in early development and subsequent lethality (Chen et al., 2012; Rienzo & Casamassimi, 2016; Mendoza-Figueroa et al., 2020).

In vivo/in vitro model system with the targeted gene deleted provide researchers an important tool for studying gene function. Genome editing technologies, such as ZFN, TALEN and CRISPR/Cas9 systems, have emerged as powerful tools for characterizing gene functions, holding significant potential for applications in human gene therapy (Ansai & Kinoshita, 2014). Depletion of integrator subunits, including *IntS12*, has a minimal impact on the 3'-end processing of snRNAs in both *Drosophila* and human HeLa cells (Mendoza-Figueroa et al., 2020). Therefore, creating an *INTS14* knockout animal model is highly significant for studying its impact on the INT in transcription and developmental processes.

In this study, we initially examined the expression patterns of the *ints14* gene in various cell types and tissues during developmental stages using zebrafish. We generated for the first time an *ints14* mutant in zebrafish, using CRISPR/Cas9 technology, as a model to study developmental disease. A zebrafish mutant model for *ints14/vwa9* gene would be an effective tool to investigate the underlying mechanisms during the processing and expression of RNA PolIII with integrator-related genes in the developmental phenomenon.

MATERIALS AND METHODS

1. Zebrafish maintenance

Zebrafish were maintained as described in a previous study (Aoyama et al., 2015). Wild-type embryos were obtained by breeding males and females. All animal studies were approved by the Animal Welfare and Research Ethics Committee at the National Marine Biodiversity Institute of Korea (MAB-23-03), and all procedures were conducted strictly in accordance with the Guide for the Care and Use of Laboratory Animals. All surgery was performed under anesthesia, and every effort was made to minimize animal suffering.

2. Reverse-transcription PCR analysis of *INTS14* mRNA

Total RNA was extracted using the TRIzol reagent (Ambion, Austin, TX, USA), and the RNA was quantified with a spectrophotometer (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from total RNA (1 µg) with a SuperScript IV first-strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA). The *ints14* cDNA was amplified with real-time PCR, using the *ints14* forward primer (5'-CATAACTGTTCTTCAGCGTGAAA-3') and *ints14* reverse primer (5'-CACTCCTGCTGTACTACATTACT-3'). *Beta-actin* cDNA was amplified with PCR, using the *b-actin* forward primer (5'-CGAGCTGTCTTCCCATCCA-3') and *b-actin* reverse primer (5'-TCACCAACGTAGCTGTCTTTCTG-3'). The reaction conditions were as follows: 95°C, 5 min; 33 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and then 72°C, 5 min. PCR product size was estimated via electrophoresis on a 1.2% agarose gel.

3. Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization (WISH) was performed as previously described (Jung et al., 2012). Antisense DIG-labeled riboprobes for *INTS14* were synthesized from linearized plasmids by using Sp6 RNA polymerase (Ambion). Photos images were taken with a stereo zoom microscope (SMZ25, Nikon, Tokyo, Japan).

4. Construction of guide RNAs and microinjection

sgRNA (5'-ACGCGACCGGTGTCAGTGGAGGG-3') of *ints14* (accession number. NM_199834) was generated and designed by using Toolgen (Seoul, Korea). Cas9 protein was purchased from Toolgen. *Ints14* target sequences are located in exon 2. *Ints14* sgRNAs (500 ng per 1 µL) and Cas9 protein (1 µg per µL) with 2 µL of 0.5% phenol red were mixed and injected into 1-cell stage embryos, which were subsequently raised to adulthood.

5. Genomic DNA preparation

Adult zebrafish were anesthetized with 0.6 mM Tricaine solution (Sigma-Aldrich, St. Louis, MO, USA). Zebrafish caudal fins were amputated with a surgical scalpel blade (Cat. HSB 704-10, Hammacher, Solingen, Germany). For genomic DNA preparation, amputated caudal fins of adult fish or whole single embryos at 1–3 dpf (day post-fertilization) were lysed essentially as described [18]. The lysates [50 mM NaOH, 1M Tris-HCl (pH 8.0)] were used for PCR amplification.

6. Genotyping analysis

PCR amplification of the targeting sequences in the *ints14* gene for 436 bp PCR products was performed with HiPi DNA polymerase (Elpis, Daejeon, Korea) via PCR using the following primers: *ints14* exon 2 forward primer (5'-CATAACTGTTCTTCAGCGTGAAA-3') and *ints14* exon 3 reverse primer (5'-CACTCCTGCTGTACTACATTACT-3'). The reaction program comprised the following steps: 95°C for 5 min, 33 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min. The PCR products were gel purified and assayed using T7E1 enzyme (Toolgen), cloned into the pGEM T-easy vector (Promega, USA), and sequenced to determine the exact sequences (Macrogen, Seoul, Korea).

RESULTS AND DISCUSSION

We aimed to observe the spatiotemporal expression of *ints14* at different stages of zebrafish embryo development by using reverse transcription polymerase chain reaction (RT-PCR) and WISH. As shown in Fig. 1A, *ints14* expression, detectable in the maternal gene expression stage,

remained constant from 6 to 96 hpf (hour post fertilization). WISH data showed that *ints14* mRNA was expressed ubiquitously from the blastula stage to segmentation (Fig. 1B). In 24 hpf embryos, *ints14* was strongly expressed in the brain and eye and weakly expressed in the trunk and tail region. In 48 hpf embryos, *ints14* was expressed in the brain and yolk sac. At 48 hpf, *ints14* expression was maintained in the brain. Spatiotemporal expression pattern analysis showed that *ints14* functions in various cells from the early developmental stage. Thus, it can be confirmed that *ints14* affects brain development at later stages.

The INTS14/VWA9 protein is a multifunctional protein complex that consists of 14 subunits referred to as Int1–Int14 (INT subunits). This complex plays a crucial role in determining the fate of numerous nascent RNAs transcribed by RNAPII. Multiple individuals with severe neurodevelopmental delay and cancers that carry biallelic mutations in integrator subunits have now been identified (Mendoza-Figueroa et al., 2020). Genetic disease models in the *ints14* gene have not been reported to date. Thus, there is an unmet need to generate *ints14* gene knockout zebrafish to model this phenomenon. The zebrafish *ints14* gene (GenBank accession number: NM_199834.1) is located on zebrafish chromosome 18. Twelve exons have been identified, with the ATG start codon in exon 2 and the TAG stop codon in exon 12. Exon 2 was selected as a target site. We designed a sgRNA targeted on exon 2 of the zebrafish *ints14* genome (Fig. 2, Table 1). It appears that the designed and produced target sequence sgRNA containing the NGG protospacer adjacent motif required for Cas9 cleavage will not have the activity of binding to any other site in the zebrafish genome.

To generate *ints14* zebrafish mutant utilizing CRISPR/Cas9 technology, we tested the efficiency for sgRNA by co-injecting with Cas9 protein into one-cell stage embryos. After performing DNA

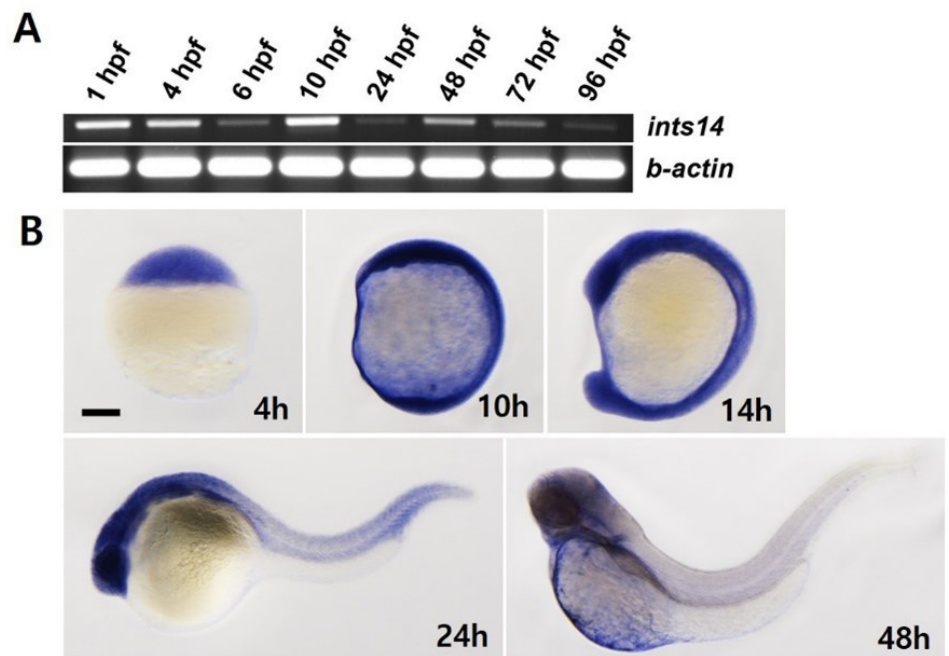


Fig. 1. Stage-dependent expression of zebrafish *ints14* mRNA. (A) RT-PCR analysis of *ints14* expression in developing zebrafish embryo. The number indicates different development stages as hour post fertilization (hpf). Embryos of indicated stages were collected and applied to RT-PCR using *beta-actin* as internal control. (B) Whole-mount *in situ* hybridization analysis of *ints14* expression in zebrafish embryo at the indicated developmental stage. All panels are dorsal, top, or lateral views with animal poles up or anterior to the left. Scale bars indicate 250µm. RT-PCR, reverse transcription polymerase chain reaction.

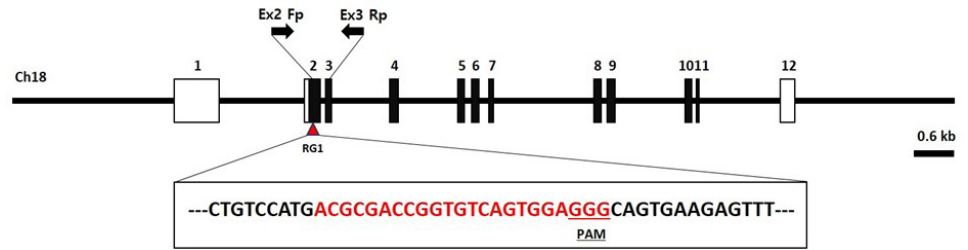


Fig. 2. Schematic representation of sgRNA (RG1) specific to exon 2 and primers for PCR genotyping of the zebrafish *ints14* locus in chromosome 18. Nucleotide sequence in exon 2 indicating the location and sequence of gRNA target site, predicted double-stranded break (DSB) (red word), and protospacer adjacent motif (PAM) (GGG, red).

Table 1. sgRNA sequence

Gene	Accession number	RGEN name	Sequence (5'-3')	Region
<i>ints14</i>	NM_199834	RG1	ACGCGACCGGTGTCAGTGGAGGG	Exon 2

sequencing and T7E1 assay for mismatch of target sequences in co-injected embryos, we found that the sgRNA efficiently generated mismatched DNA for the target region, suggesting successful generation of the *ints14* mutant zebrafish (Fig. 3, Table 2).

The Cas9 protein and single sgRNA were co-injected into the cytoplasm of zebrafish fertilized eggs. Founder fishes injected with *ints14* and Cas9 were crossed with wild-type to obtain 1–3

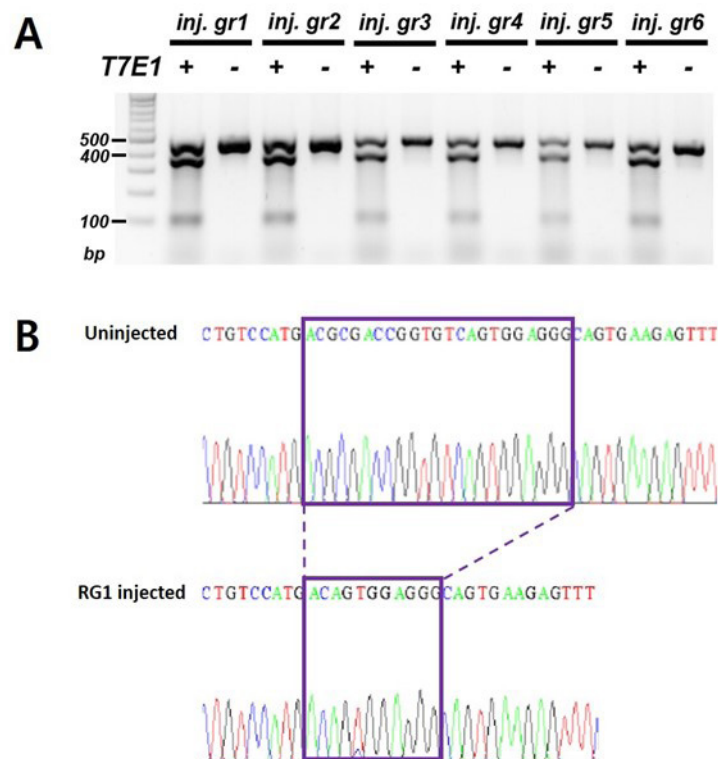


Fig. 3. Test of activity for *ints14* RGEN in zebrafish. (A) To identify the efficiency of the sgRNA, we co-injected *ints14* sgRNA (RG1) and Cas9 protein. Co-injected embryos showed that the sgRNA efficiently generated mismatched DNA when the T7 endonuclease I (T7E1) assay was performed. (B) The TA-cloning and sequencing results demonstrate that a deletion of approximately 12 bp can be achieved via the RGEN system.

Table 2. Primers and product size for PCR genotyping and T7E1 assay

Target of sgRNA	PCR size	T7E1 assay		ID primer	Sequence
<i>ints14</i> exon2	436 bp	336±@ bp	100±@ bp	Ex2 Fp	CATAACTGTTCTTCAGCGTGTA
				Ex3 Rp	CACTCCTGCTGTACTACATTACT

days embryo. To confirm the disruption of *ints14* gene in these fish, PCR and T7E1 assays were performed (Table 2). Compared with wild-type zebrafish, the desired fragment deletion of *ints14* gene spanning the sgRNA site was detected by using T7E1 assay. A clear band demonstrates the fragment deletion of *ints14* in #2 and #4 lines of the zebrafish F1 generation (Fig. 4A). The TA-cloning and sequencing results demonstrate that a deletion of approximately 9 or 6 bp can be achieved via the single gRNA system (Fig. 4B and C). These frameshift mutations of *ints14* resulted in termination site change and early termination of the INTS14 protein, indicating that the INTS14 protein was abnormally generated. However, no lethality phenomenon was observed in the early embryonic stages of the *ints14* knockout model. In the future, there is a need for research on behavioral and developmental abnormalities during the later stages. While the molecular mechanisms of *ints14* have been reported, future research is anticipated to explore its cellular and developmental functions using the model we have established. The investigation into the interplay of INTS14 with cellular processes and its potential associations with human diseases holds promise for advancing our understanding of its broader implications.

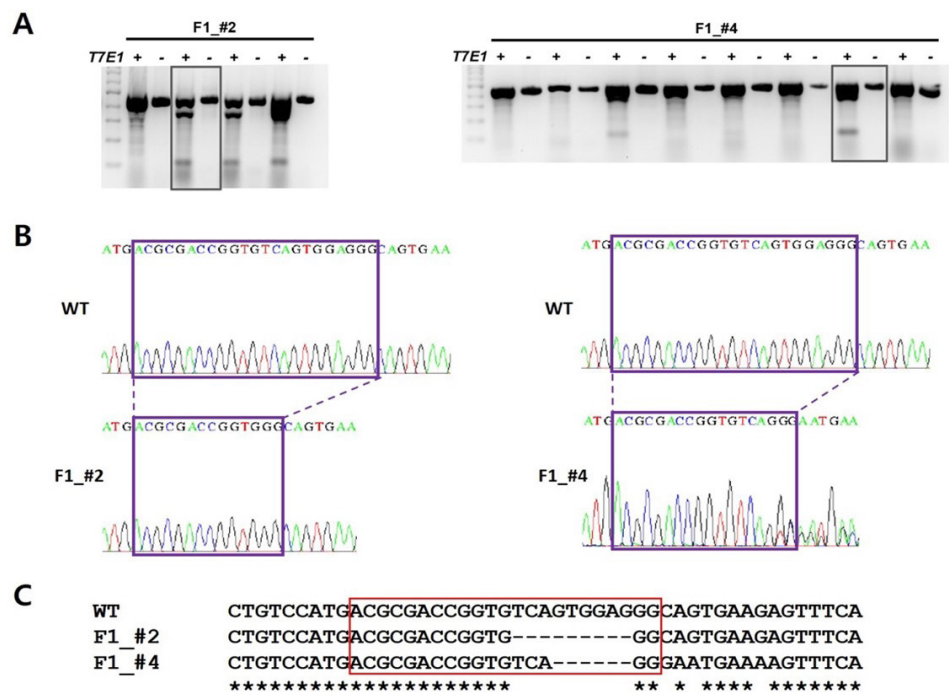


Fig. 4. Generation of *ints14* knockout zebrafish model using CRISPR/Cas9 gene editing technology. (A) F0 individuals, upon reaching adulthood, transmitted the *ints14* knockout mutation to their offspring, illustrating a high efficiency of targeted mutagenesis for the established lines #2 and #4 by T7E1 assay. (B, C) Results of sequencing found that F1 #2 and F1 #4 individuals exhibited 9 and 6 bp deletions, respectively; thus, frameshift mutation in *ints14* led to a modification of the termination site, resulting in the early termination of the *ints14* protein.

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