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Identification of Gin-α Hyperactivated Recombinase-based RNA-Guided Recombinase Platform Target Sites in the Zebrafish Genome

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ABSTRACT

Background: RNA-guided recombinase (RGR) platform is recombinase action-based genome engineering platform. It is a fusion of dead Cas9 (dCas9) and evolved recombinase with relaxed target specificity. RGR is facile, programmable and has high genome targeting capacity and a safer alternative to conventional nuclease-based methods, which carry a risk of genotoxicity. The present study was conducted to map the entire zebrafish genome for the locations of Gin-α hyperactivated recombinase-based RGR platform target sites with varying spacer lengths.

Methods: RGR platform target site DNA patterns with varying spacer lengths (5/6/7-base pair) were searched in all zebrafish chromosomes using the NCBI Genome Data Viewer (GDV) genome browser. Genomic contexts of the located RGR platform target sites were further documented using NCBI Blast.

Result: The study identified 262 RGR platform target sites with 5-bp spacer, 307 sites with 6-bp spacer and 326 sites with 7-bp spacer. It also analyzed the genomic context of these sites, identifying zebrafish genes located near the identified target sites. This work contributes to the development of genome editing techniques that are highly specific and safer, as well as programmable and simple and provides tools to utilize them in model organisms like zebrafish, which are commonly used in various genetic and biomedical studies.

Key words: Hyperactivated recombinase, RGR platform, Safer genome editing, Zebrafish.

INTRODUCTION

Genome editing (GE) allows for precise modifications to the DNA of cells or organisms, enabling the addition, removal, or alteration of genetic material (WHO, 2024). Modern GE technologies, such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas9, have revolutionized the field, making it possible to cure genetic disorders also (Xie *et al*., 2014; Ul Ain *et al*., 2015). These tools typically rely on nuclease action, which induces double-strand breaks (DSBs) in target genomic DNA, followed by error-prone cellular repair, leading to genome modification (Silva *et al*., 2011). However, conventional nuclease-based GE methods pose risks, including genotoxicity due to DSBs and off-target effects that cause unintended mutations (Gabriel *et al*., 2011; Fu *et al.,* 2014; Boutin *et al*., 2022). These methods are also dependent on cellular repair processes (Sancar *et al*., 2004). Recombinase-based GE provides a safer alternative, performing precise DNA modifications without DSBs and reducing genotoxicity risks. Recombinases, such as site-specific recombinases (SSRs), result in a single, defined outcome (Grindley *et al*., 2006). SSRs are classified into tyrosine recombinases (*e.g.*, Flp, Cre) and serine recombinases (*e.g.*, Hin, Sin, Gin), with serine recombinases offering more flexibility for genome engineering (Grainge and Jayaram 1999; Grindley *et al*., 2006; Sarkar *et al*., 2007). The use of wild site-specific recombinases (SSRs) in genome editing (GE) has been limited by their rigid target specificity, requiring rare pre-existing sites in higher eukaryotic genomes or labor-intensive pre-introduction of target sites therein (Thyagarajan *et al*., 2000; Chalberg *et al*., 2006; Grindley *et al*., 2006). Additionally, wild SSRs face challenges related to topological and spatial constraints due to the need for accessory proteins (Smith and Thorpe, 2002; Sancar *et al*., 2004).

To address these issues, 'hyperactivated' SSR variants with relaxed target specificity and non-requirement of accessory proteins have been developed through techniques like directed evolution. These variants can recombine minimal recognition sequences, expanding their applicability in GE (Buchholz and Stewart, 2001; Gaj *et al*., 2013; Sirk *et al*., 2014). The RGR platform-a fusion of

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dead Cas9 (dCas9) and hyperactivated SSR-further simplifies this by using the CRISPR/Cas9 system to guide recombination, making genome editing facile and programmable (Mali *et al*., 2013; Sonwane, 2014; Chaikind *et al*., 2016; Standage-Beier *et al*., 2019; Sonwane *et al*., 2022).

We have developed such a RGR platform based on Gin-α hyperactivated recombinase previously developed by Gaj *et al*. (2013) and evaluated it for targeted DNA integration in human and bovine genomes (Pathak, 2020; Sonwane 2021). This RGR platform functions on a lengthy seventy-six to eighty base pair target sites comprised of a degenerate central core recombinase recognition sites (20-bp) flanked by spacer sequences (5/6/7-bp), that are flanked by gRNA binding sites (20-bp); this complete region is flanked by protospacer adjacent motif (PAM) sequences (3-bp). The sequence of this RGR platform target site is 5′-$CCN_{(30-32)}$AAABNWWNVTTTN$_{(30-32)}$GG-3′ (Sandeep, 2021; Sonwane, 2021).

Zebrafish are a key model for genetic and biomedical research due to their high genetic similarity to humans, ease of manipulation and suitability for large-scale studies (Teame *et al.,* 2019; Espino-Saldaña *et al*., 2020; Choi *et al*., 2021). Advanced genome editing technologies like CRISPR/Cas9 have significantly improved zebrafish research, facilitating the modeling of human diseases (Doyon *et al*., 2008; Bedell *et al*., 2012; Varshney *et al*., 2015). In light of these advancements, mapping the zebrafish genome for novel, safer genome engineering platform like RGR platform target sites is essential for developing tools like path activity reporters, which are crucial for studying complex biological processes. This study offers a comprehensive map of Gin-α hyperactivated recombinase-based RGR platform target sites with spacer lengths of five, six and seven base pairs across the zebrafish genome, along with the information of genes located near these sites.

Materials and Methods

This research was conducted at the Fish Genetics and Biotechnology Laboratory of ICAR-Central Institute of Fisheries Education, Mumbai as a part of Ph.D. research work from 2020 to 2023.

Identification of Gin-α hyperactivated recombinase-based RGR genome editing platform target sites in zebrafish genome

DNA patterns to search locations of the RGR platform target sites were designed as per the structures of the target sites. The DNA pattern for seventy-six base pair Gin-α recombinase-based RNA-Guided Recombinase (RGR) genome editing platform target sites with 5-base pair spacer sequence (5′-CCN_{30} AAABNWWNVTTTN$_{30}$GG-3′), for seventy-eight base pair target sites with 6 base pair spacer sequence (5′-CCN_{31} AAABNWWNVTTTN$_{31}$ GG-3′) and for eighty base pair target sites with 7-base pair spacer sequence (5′CCN_{32} AAABNWWNVTTTN$_{32}$GG-3′) where N = [ATGC], B = [TCG], W = [AT] and V = [ACG].

For locating RGR platform target sites in zebrafish genome, NCBI Genome Data Viewer (GDV) genome browser (https://www.ncbi.nlm.nih.gov/gdv/) was used. Chromosome-wise sequence data for zebrafish was accessed by searching the browser for zebrafish (*Danio rerio*) genome data. Recent zebrafish genome data assembly GRCz11 was selected for this purpose. Each chromosome was then selected one at a time to locate RGR platform target sites on it. Then to locate RGR platform target sites on the chromosome, one of the three above-designed DNA patterns was searched using the 'Tools' menu. This resulted in the display of all the sequences that conform to the input DNA pattern. All the sequences were saved and several found out target sites per chromosome were documented.

Documenting zebrafish genes 'in context' with the RGR platform target sites

For this purpose, all identified RGR platform target site sequences were converted into Fasta format. Then, by using blastn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearchandLINK_LOC=blasthome) of NCBI (National Center for Biotechnology Information), RGR target site sequences were mapped against RefSeq Genome Database (RefSeq genomes) of *Danio rerio* (Taxid: 7955) for highly similar sequences. Genomic sequences (Subject sequences) having 100% alignment with no gap with RGR target site sequences (Query sequences) were selected and features of each site were documented to determine whether the given site is 'in context' with any gene(s).

Results and Discussion

Identification of Gin-α hyperactivated recombinase-based RGR genome editing platform target sites in zebrafish genome

This study mapped target sites for the Gin-α hyperactivated recombinase-based RGR platform in the zebrafish genome, identifying 262 sites with a 5-base pair spacer length, 307 sites with a 6-base pair spacer length and 326 sites with a 7-base pair spacer length. The chromosome-wise distribution of these RGR platform target sites is detailed in Table 1. Among the 262 target sites with a 5-base pair spacer, 184 were located within genic regions and 78 in intergenic regions. For the 307 sites with a 6-base pair spacer, 190 were genic and 117 were intergenic. The 326 sites with a 7-base pair spacer included 242 genic and 84 intergenic sites. Table 2 presents the genic and intergenic distribution of these sites.

Documenting zebrafish genes 'in context' with the RGR target sites

In the present study a number of RGR target sites were discovered to be present in several protein-coding genes, with significant biological actions. These include members

of the kinesin family, *1Ab-kif1ab*; regulator of G protein signalling 2, *gpsm2*; leucine-rich repeat and coiled-coil centrosomal protein 1, *lrrcc1*; testis-specific kinase, *tesk2*; guanine nucleotide-binding protein (G protein), *gamma 13b*; membrane-associated ring finger (C3HC4) 7; ret proto-oncogene receptor tyrosine kinase; nuclear receptor coactivator 4; polymerase (DNA directed) eta; tumour protein p53 inducible protein 11b; chromodomain helicase DNA binding protein 4a; fibronectin type III domain containing 5b; collagen, type VIII, alpha 2; elongation factor RNA polymerase II; *etc.*

Present study identified Gin-α hyperactivated recombinase-based RGR platform target sites in entire genome of zebrafish vertebrate animal model. It further documented all the genes those are located near these identified target sites. Previous studies mapped the genomes of various organisms for RGR platform target sites. Pathak *et al*. (2020) identified 436 target sites for an RGR platform using hyperactivated recombinase β with a 5-base pair spacer length in the bovine genome. In a subsequent study, 677 RGR target sites were found in the bovine genome using hyperactivated recombinase Gin-α with a 6-base pair spacer (Pathak *et al*., 2022). Similarly, Tarang *et al*. (2023) mapped 426 target sites in the buffalo genome for an RGR platform based on hyperactivated recombinase β with a 6-base pair spacer. The current study extends this work by locating target sites for an RGR platform based on hyperactivated recombinase Gin-α with spacer lengths of 5, 6 and 7-base pairs in the entire zebrafish genome. The RGR platform is designed to accommodate spacer lengths of 5-7-base pairs, flanking the 20-base pair central core recombinase binding site. This flexibility in spacer length enhances the genome targeting capacity of the RGR platform. Consequently, this study identifies numerous RGR target sites across the entire zebrafish genome.

Table 1: Chromosome-wise distribution of next-generation Gin-α hyperactivated recombinase-based RNA-Guided Recombinase (RGR) Genome Editing platform target sites in zebrafish genome.

Chromosome	Size (MB)	5-bp spacer	6-bp spacer	7-bp spacer
1	17	14	14	17
2	17	5	15	20
3	18	21	15	16
4	21	5	25	13
5	21	22	13	22
6	17	11	17	14
7	21	20	16	16
8	16	13	18	10
9	16	13	16	17
10	13	3	8	12
11	13	17	11	13
12	14	7	9	8
13	15	14	14	15
14	15	2	8	16
15	14	6	6	14
16	16	9	12	10
17	15	6	8	15
18	15	6	13	12
19	14	13	8	17
20	16	9	12	6
21	13	4	10	9
22	11	9	9	7
23	13	12	14	14
24	12	14	10	7
25	11	7	6	6

An RGR site in gene kinesin family member 1Ab was located on chromosome 2. This gene is related to vesicle-mediated transport, microtubule-based mobility and intracellular transport depending on the cytoskeleton. Human homolog KIF1A is linked to neurological disorders; zebrafish with a KIF1A mutation showed altered synaptic function and seizure activity (Guo *et al*., 2020). One RGR platform target site within the gene guanine nucleotide-binding protein (G protein) gamma 13b was identified on zebrafish chromosome 3. This gene facilitates the binding activity of G-protein beta-subunits and plays a crucial role in the G protein-coupled receptor (GPCR) signalling cascade, akin to the human GNG13 (gamma 13 G protein component). Studies suggested that, mutations affecting prenylation of G protein gamma subunits disrupt signalling in zebrafish (Mulligan *et al*., 2010).

On chromosome 13, a target site for RGR was found within the ret proto-oncogene receptor tyrosine kinase gene. This gene encodes a transmembrane receptor belonging to the tyrosine-protein kinase family. Mutations are associated with Hirschsprung Disease and central hypoventilation syndrome; a zebrafish model has been used to study these effects (Heanue *et al*., 2016). An RGR platform target site within the gene polymerase (DNA-directed) eta was detected on chromosome 13. This gene facilitates the activity of DNA-directed DNA polymerase and is involved in pyrimidine dimer repair, post-replication repair and the cellular response to UV-C. It is expected to be localized in the nucleoplasm and cytosol. The human ortholog of this gene, POLH (DNA polymerase eta), is associated with a specific form of xeroderma pigmentosum (Eckert, 2023). On chromosome 2, another RGR site was

Table 2: Genic and intergenic distribution of the RGR target sites in the zebrafish genome.

RGR Target site	Genic sites	Intergenic sites
RGR target sites with 5-base pair spacer length	184	78
RGR target sites with 6-base pair spacer length	190	117
RGR target sites with 7-base pair spacer length	242	84

detected within the leucine-rich repeat and coiled-coil centrosomal protein 1 (*lrrcc1*) gene. This gene encodes a pivotal centrosomal protein essential for forming the mitotic spindle and maintaining centrosome structure. Its encoded protein comprises an N-terminal leucine-rich repeat domain and a C-terminal coiled-coil domain. Additionally, an RGR platform target site was pinpointed within the collagen, type VIII (*col8a1a*) gene, also situated on chromosome 2. Type VIII collagen functions as a structural element in the extracellular matrix, influencing head and notochord development. Gray *et al*. (2014) found that impaired *col8a1a* activity during zebrafish development leads to congenital vertebral anomalies, indicating its critical role in embryogenesis.

An RGR platform target site was identified in the chromodomain helicase DNA binding protein 4a gene on chromosome 19. This protein, part of the SNF2/RAD54 helicase family, is involved in histone deacetylase binding, ATP and RNA polymerase II interactions and fin regeneration. It plays a role in epigenetic transcriptional repression and is linked to dermatomyositis and serous endometrial tumours (Wang *et al*., 2020; Novillo *et al*., 2021). Similar to the human CHD5, zebrafish Chd5 is crucial for neurogenesis and tumour suppression. Knockdown of Chd5 in zebrafish causes craniofacial defects and affects neural development (Bishop *et al*., 2015). Another RGR target site was found in the fibronectin type III domain containing 5b gene on chromosome 19. FNDC5, which encodes the myokine *irisin*, is involved in muscle function, energy expenditure and appetite regulation. Knockdown of *irisin* in zebrafish leads to reduced appetite and altered peptide levels (Sundarrajan and Unniappan, 2017).

Traditional genome engineering tools like ZFNs, TALENs and CRISPR-Cas9 involve double-strand DNA breaks and are dependent on cellular DNA repair, which can lead to risks and inefficiencies. Recombinase-based genome engineering tools offer a safer alternative by avoiding DNA breaks and repair machinery dependencies. Recent advancements include hyperactivated recombinases with broad targeting capabilities. Combining these with modular DNA targeting domains like zinc-fingers or TALEs has led to the development of zinc-finger recombinases (ZFRs) and TALE recombinases (TALERs).

The CRISPR-Cas9 system, when used as dead Cas9 (dCas9), can be coupled with hyperactivated recombinases to create a programmable RNA-guided recombinase (RGR) platform for precise genome editing. This system has been utilized for targeted DNA integration in humans and cows (Sonwane, 2021). For zebrafish, the RGR platform was tested for targeted transgenesis. A permissive genomic locus, orthologous to the mouse *Enah-Srp9* intergenic region, was identified for efficient transgene integration, achieving a 46.8% transgenesis rate with consistent expression patterns across different zebrafish body regions (Padhan, 2022; Nidarshan, 2023) in this.

Conclusion

The RNA-guided recombinase (RGR) platform combines dead Cas9 (dCas9) with hyperactivated recombinases that have relaxed target specificity, functioning as a dimer. Its genomic target sites are typically 76 to 80 bp in length. Mapping these sites across the genome is crucial for leveraging this next-generation genome engineering tool. This study provides a comprehensive map of RGR platform target sites with spacer lengths of five, six and seven base pairs in the zebrafish genome. This detailed mapping will facilitate precise and safer DNA modifications such as integration, deletion and inversion in zebrafish models, enhancing the utility of the RGR platform for future genetic research and applications.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not represent the views of their affiliated institutions. We are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

Not applicable.

Conflict of interest

All the other authors declare that they have no competing interests.

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