Send Orders for Reprints to reprints@benthamscience.net

REVIEW ARTICLE

Modeling Neuronal Diseases in Zebrafish in the Era of CRISPR

Angeles Edith Espino-Saldaña[1,3], Roberto Rodríguez-Ortiz[2], Elizabeth Pereida-Jaramillo[1] and Ataúlfo Martínez-Torres[1,*]

[1]*Departamento de Neurobiología Celular y Molecular, Laboratorio de Neurobiología Molecular y Celular, Instituto de Neurobiología, Campus UNAM Juriquilla, Querétaro, Qro CP76230, México;* [2]*CONACYT - Instituto de Neurobiología, Universidad Nacional Autónoma de México. Querétaro, Qro., México;* [3]*Universidad Autónoma de Querétaro, Facultad de Ciencias Naturales, Av. de las Ciencias S/N, Querétaro, Mexico*

ARTICLE HISTORY

Received: April 03, 2019
Revised: July 04, 2019
Accepted: September 29, 2019

DOI:
10.2174/1570159X17666191001145550

Abstract: ***Background***: *Danio rerio* is a powerful experimental model for studies in genetics and development. Recently, CRISPR technology has been applied in this species to mimic various human diseases, including those affecting the nervous system. Zebrafish offer multiple experimental advantages: external embryogenesis, rapid development, transparent embryos, short life cycle, and basic neurobiological processes shared with humans. This animal model, together with the CRISPR system, emerging imaging technologies, and novel behavioral approaches, lay the basis for a prominent future in neuropathology and will undoubtedly accelerate our understanding of brain function and its disorders.

Objective: Gather relevant findings from studies that have used CRISPR technologies in zebrafish to explore basic neuronal function and model human diseases.

Method: We systematically reviewed the most recent literature about CRISPR technology applications for understanding brain function and neurological disorders in *D. rerio*. We highlighted the key role of CRISPR in driving forward our understanding of particular topics in neuroscience.

Results: We show specific advances in neurobiology when the CRISPR system has been applied in zebrafish and describe how CRISPR is accelerating our understanding of brain organization.

Conclusion: Today, CRISPR is the preferred method to modify genomes of practically any living organism. Despite the rapid development of CRISPR technologies to generate disease models in zebrafish, more efforts are needed to efficiently combine different disciplines to find the etiology and treatments for many brain diseases.

Keywords: Brain disease models, *Danio rerio*, genome engineering, zebrafish, optogenetics, CRISPR.

1. INTRODUCTION

Since the 1980s, zebrafish (*Danio rerio*) has quickly become a popular model in biological research to study genetics and developmental biology [1]. Since then, several techniques have made zebrafish one of the fastest-growing experimental model organisms, with great potential for new discoveries. Recently, the novel CRISPR/Cas system (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein) has allowed the successful introduction of mutations to the genome or control gene expression in zebrafish. Here, we review the most recent studies about CRISPR technologies applied for understanding brain function and neurological disorders in *D. rerio*.

*Address correspondence to this author at the Departamento de Neurobiología Celular y Molecular, Laboratorio de Neurobiología Molecular y Celular, Instituto de Neurobiología, Campus UNAM Juriquilla, Querétaro, Qro CP76230, México; Tel/Fax: +52 442 238 1064;
E-mail: ataulfo@unam.mx

2. FEATURES AND ADVANTAGES OF THE ZEBRAFISH EXPERIMENTAL ANIMAL MODEL

Several features make zebrafish an ideal system to perform studies in biology. A female can release up to 100–200 eggs that are externally fertilized. Embryos develop rapidly, and most organs are functional within a few days after fertilization [2]. Because the embryos are transparent during early embryogenesis, individual cells and processes can be visualized *in vivo*, making it easy to monitor the dynamics of gene expression in various tissues and organs without the need to sacrifice the experimental subjects. Zebrafish maturation takes only 2 ~ 3 months, which consequently saves time for generating transgenic lines. The zebrafish brain regenerates easily, allowing researchers to study mechanisms of neuroprotection and neurogenesis [3]. The organs of this species have been well-characterized, letting many fundamental questions about vertebrate biology to be addressed [4].

1570-159X/20 $65.00+.00 ©2020 Bentham Science Publishers

Zebrafish and mammalian brains share broad anatomical and functional features, such as the presence and main functions of the cerebellum, diencephalon, amygdala, spinal cord, and enteric-autonomic nervous systems, although it is uncertain whether the zebrafish telencephalon is functionally equivalent to the mammalian telencephalon [5-10]. They share conserved neurotransmitter systems, such as GABA (γ-Aminobutyric acid), glutamate, dopamine, serotonin, noradrenaline, histamine, and acetylcholine [6]. Additionally, organogenesis in zebrafish is remarkably similar to that of humans [11]. These features make the zebrafish an excellent vertebrate model for studying human diseases, including neuronal disorders [12-15]. Furthermore, potential therapeutic drugs that target processes in various diseases have been discovered because chemical screening in zebrafish is straightforward and cost-effective [16, 17]. Using zebrafish larvae is considered by some researchers as more ethically acceptable than using rodents in biomedical research [18].

The current zebrafish genome assembly (GRCz11) has 25,592 coding genes and 6,599 non-coding genes resulting in 59,876 gene transcripts ("Zebrafish assembly and gene annotation" recovered from http://www.ensembl.org/Danio_rerio/Info/Annotation, last updated in April 2018). A comparison to the human reference genome shows that 71.4% of human genes have at least one obvious zebrafish ortholog [19]. The high number of gene transcripts compared to the coding genes derives mainly from alternative splicing events, which leads to large protein variability. Genomic analysis of human, mouse, and zebrafish suggested that gene duplication followed by exon structure divergence between paralog genes caused a significant reduction of alternative splicing [20].

Zebrafish have been successfully used to understand the biological activity of gene orthologs to human disease-related genes [14]. Out of all the human genes bearing morbidity descriptions listed in the Online Mendelian Inheritance in Man database, 82 % can be related to at least one zebrafish ortholog [19]. ZFIN, the Zebrafish Information Network (http://www.zfin.org), is the primary repository of information related to zebrafish genetics and genomics. ZFIN displays a variety of data types for each gene, such as sequence features, different available alleles, sequence targeting reagents including CRISPR guide RNA sequences; transgenics, phenotypes, expression, available antibodies, ontologies, homologues, and links to external databases, nomenclature, publications, and figure images.

3. MUTAGENESIS AND KNOCKDOWN IN ZEBRAFISH BEFORE CRISPR

Since the early 1990s, various protocols have been developed to allow successful gene editing and accurate control of gene expression of the zebrafish genome. First, chemical mutagenesis was used to perform random screens [21]. Shortly after, a method for generating transgenic fish using pseudotyped retroviruses was developed [22]. With this type of retrovirus, another large screen for developmental defects was achieved [23, 24] with the advantage that DNA integration events allowed all mutated genes to be systematically identified. In the late 1990s, transposable elements were described to be active in the zebrafish genome; the Tol2 transposons, which were part of a procedure consisting of microinjecting single-cell embryos with the mRNA of the transposase and a number of vectors with specific features and applications [25]. Later, Sleeping Beauty and Ac/Ds transposons were also used [26, 27]. These genetic tools and techniques have made it possible to identify essential genes and their localization, introduce mutations to these genes, and generate a wide variety of knockin transgenic zebrafish lines, thereby providing multiple potential applications for functional genomic studies [28, 29]. An example of this technology in the fish genome is the introduction of an engineered TRPV4-ferritin chimera that allowed the magnetic control of neural function [30].

New technologies for targeted mutagenesis take advantage of genome-editing nucleases. These approaches allow scientists to generate mutations in specific genes or sites of the genome. In 2008, ZFNs (zinc finger nucleases) were demonstrated to be effective in zebrafish [31, 32]; this was the first demonstration of specifically targeted gene inactivation in this model. While ZFNs were effective tools for making targeted mutations, they required developing significant expertise to assemble them properly, or if commercially purchased, the cost was considerably high. In 2011, TALENs (Transcription activator-like effector nuclease) were adapted to zebrafish gene targeting [33]. The relatively modest cost of reagents compared with ZFN and ease of assembly made TALENs a more attractive alternative to ZFNs for large-scale targeted gene disruption [34]. Although this method has been proved to be effective, some TALEN pairs provide little to no mutagenic activity and their efficiency remains variable. These techniques are falling into disuse, yet recently published works still use them [35-37].

All the techniques described above were envisioned for the modification of the zebrafish genome. However, it is sometimes necessary only to transiently knockdown gene expression. In 2000, a new technique using antisense morpholinos oligonucleotides (MOs) was shown to be effective in zebrafish. MOs are modified oligonucleotides that inhibit the translation of target mRNA *in vivo*. They can be delivered efficiently by injecting them into the yolk of one-cell stage embryos [38]. This method became popular in zebrafish research because it was easy and effective. Unfortunately, MOs had major limitations and issues. Potential off-target or inexplicable phenotypes appeared, and rescue control experiments were occasionally an artifact. Nevertheless, knockdown approaches remain useful if proper control experiments are performed. MOs can also be designed to inhibit pre-mRNA splicing or block translation initiation. MOs are especially useful to knockdown multiple alleles and when large gene families are genetically redundant [39]. Interference RNA is another popular technique for gene knockdown, but in strong contrast to *Drosophila*, *C. elegans*, and mammalian cells, few successful applications of shRNA [40, 41] and iRNA [42] have been reported in zebrafish to silence gene expression. Despite concerted efforts in the field, progress in the use of iRNA technologies in zebrafish has been extremely slow.

4. CRISPR/Cas OVERVIEW

The CRISPR system has been the most revolutionary development in the biology of recent times. It was originally described as an adaptive immune system used by bacteria to defend themselves against invading viruses by recording and targeting their DNA sequences [43]. It was demonstrated that the CRISPR/Cas system is an efficient targeted mutagenesis tool for zebrafish [44] that allows targeting multiple genes simultaneously [45]. The use of the CRISPR/Cas system in zebrafish has proven to be so efficient and simple to use that it represented no less than a revolution in zebrafish research, making it possible to easily knockout any gene in the genome [4]. A single-guide RNA (sgRNA) includes a short sequence that is homologous to the DNA target followed by a "scaffold" sequence necessary for the Cas9-binding enzyme [46]. This is enough to program Cas9 to introduce double-strand breaks DSB in target DNA. These breaks are repaired by error-prone, non-homologous end-joining (NHEJ), which leads to nucleotide insertion and/or deletion (InDels) at the genomic target site resulting in in-frame amino acid deletions, insertions, or frameshift mutations leading to premature stop codons within the open reading frame (ORF) of the targeted gene. Ideally, the end result is a loss-of-function mutation within the targeted gene. To date there are several versions of the Cas9 enzyme from *Streptococcus pyogenes*, the most commonly used bacterium in genome engineering [47-49]. Cas9 has been modified to lose endonuclease activity while maintaining the ability to target specific DNA sequences. This modified enzyme called dCas9 can be fused to diverse effector domains, such as activators, repressors, or methylases, that can be used for targeted epigenome editing to specifically modify or control gene expression, or to introduce fluorescent proteins, thus providing a tool for visualizing chromosome structure or dynamics [50]. Likewise, nucleases from a variety of organisms have diverse characteristics such as a different PAM sequence or RNA-rather than DNA-targeting. An outline of these nuclease targets and functions is listed by Xu *et al*. and Wu *et al*., [51, 52].

4.1. Editing Zebrafish with the CRISPR System

The first use of CRISPR/Cas9-mediated mutagenesis in zebrafish was demonstrated a few years ago [44]. Thenceforth, dozens of papers have been published describing the use of CRISPR in zebrafish as a tool to study developmental processes and model diseases. The CRISPR/Cas9 system was shown to be six times more efficient at generating germline mutations in zebrafish compared to ZFN and TALEN [53]. A zebrafish codon-optimized Cas9 was developed [45] and reported to increase mutagenesis efficiency [54]. A new tRNA-based multiplex sgRNA expression system has been developed in zebrafish to express multiple sgRNAs efficiently [55]; there is also a platform for generating somatic point mutations with germline transmission in the zebrafish [56, 57]. Recently, other nucleases have been optimized in zebrafish, such as Cas12a temperature-controlled genome editing. Additionally, there is a new approach that enables the direct, irreversible conversion of one target DNA base into another in a programmable manner, without requiring dsDNA backbone cleavage or a donor template [58].

The general procedure for targeted mutagenesis in zebrafish using the CRISPR system is as follows: First, sgRNAs targeting the specific gene or any other site in the genome are designed. Multiple options are available for sgRNA design such as CHOPCHOP [59] and CRISPRscan, which was created based on a large-scale analysis of sgRNA mutagenesis activity in zebrafish [60]. Second, single or multiple sgRNA guides are synthesized *in vitro* together with the mRNA encoding the Cas9 protein; other Cas proteins can be used depending on the objective. Moreover, injecting Cas protein instead of Cas mRNA could improve mutagenesis efficiency [61]. Third, these sgRNAs together with the Cas mRNA or protein are injected into fertilized embryos at the one-cell stage [62, 63]. Injection dose should be standardized to improve efficiency and avoid phenotypes due to the toxic effect of the injection components. Microinjection efficiency can also improve using an automated microinjection system [62]. Each injected embryo grows as a mosaic founder fish (crispant) with various InDels in the somatic cells as the nucleases cleave the target sites over the course of multiple rounds of cell division in the developing embryos [44]. The phenotype and genotype can be analyzed within several hours or days after the injection. The phenotype should be analyzed in various crispant embryos taking into consideration that the number and localization of mutated cells will vary in each individual due to the range of severity in the phenotypes. Numerous techniques can be used for fast genotype screening such as TIDE (Tracking of Indels by Decomposition), HMA (heteroduplex mobility assay), or T7 endonuclease digestion [64-66]. The mutation is subsequently confirmed by PCR and sequencing. A ZEG device enables genotyping while keeping the embryos alive [67]. To avoid mosaicism and phenotype variability, a homozygotic mutant line can be produced by outcrossing mosaic crispants to wild-type and then inbred F1 until homozygous are found. If the homozygote is lethal, a heterozygous line can be maintained. Fig. **1** provides an overview of the main strategies for zebrafish genome engineering using the CRISPR system.

A detailed protocol for generating and genotyping mutants using CRISPR/Cas9 in zebrafish is described by Vejnar *et al*., [68] and Rafferty *et al*., [69], and a protocol using CRISPR/Cpf1 (CRISPR-associated endonuclease in Prevotella and Francisella 1) is included in a study by Fernandez *et al*. [58]. Also, a Cas9 transgenic zebrafish strain was recently constructed [70]. Compared with the traditional CRISPR injection method, this transgenic zebrafish has shown to significantly improve the efficiency of genome editing.

To generate transgenic zebrafish lines, techniques and protocols have been successfully established for exogenous DNA integration into the zebrafish genome using CRISPR [71-74]. This allows in-frame integration of fluorescent reporters, such as eGFP (enhanced green fluorescent protein), with a specific gene, as well as a precise integration of expression cassettes into the genome, thus avoiding overexpression due to the integration of many copies in the genome or integration in a zone where the chromatin could have a different pattern. Furthermore, tissue-specific gene disruption has been achieved by driving Cas9 expression with the Gal4/UAS system. In combination with Cre/loxP systems,

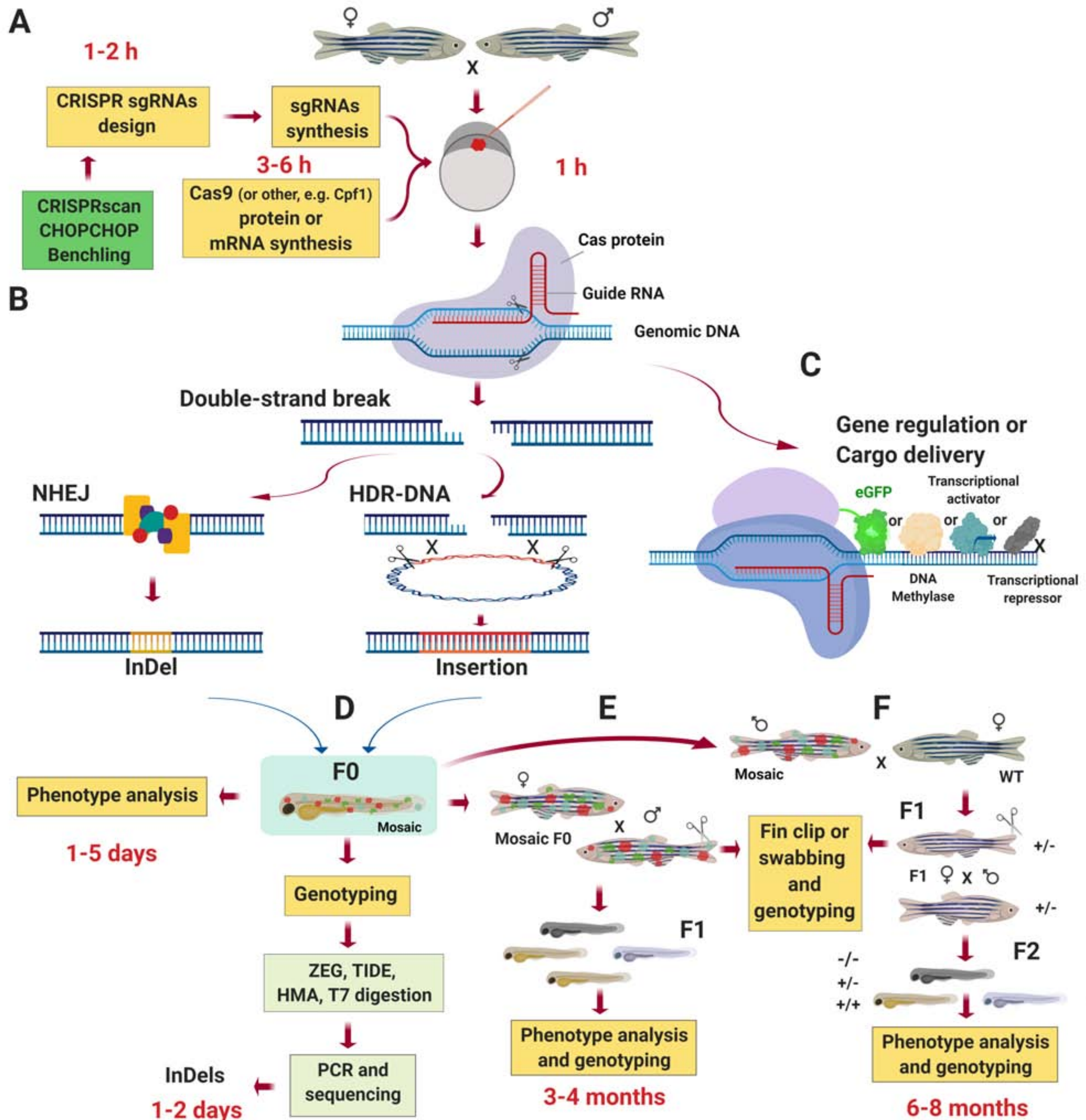

Fig. (1). Summary of the main strategies for zebrafish genome engineering using the CRISPR/Cas system. The typical time consumed in each procedure is indicated in red. **A**) sgRNA design using any of the available tools. *In vitro* synthesis of the sgRNAs and mRNA (or protein) from the Cas choice. Embryo microinjection at one-cell stage with a mix of single or multiple sgRNAs plus the following according to the purpose: for knockout, mRNA encoding Cas choice (or Cas protein); for knockin, mRNA encoding nuclease (or Cas protein) plus donor DNA; for gene regulation or cargo delivery, inactive dead Cas9 (dCas9) fused to an effector. Inside the cell, a complex is formed between the Cas protein, the sgRNA and the genomic DNA. **B**) The endonuclease generates a genomic DNA double-strand break, which is repaired by the endogenous DNA repair machinery by non-homologous end joining (NHEJ), causing insertions or deletions (InDels) that could disturb the open reading frame (knockout) or incorporate exogenous donor DNA into a homology-independent process at a chosen genomic locus (knockin). **C**) dCas9 can be used fused to an effector such as transcriptional repressors, activators, DNA methylases, or fluorescent proteins. In this case, the complex, instead of breaking the genomic DNA, allows gene regulation or target visualization. **D**) Phenotypes can be observed in the injected embryos after 1 to 5 days. Genotype screening can be performed in a portion of the mosaic F0 by a quick procedure such as TIDE (Tracking of Indels by Decomposition), HMA (heteroduplex mobility assay), or T7 endonuclease digestion, and then confirmed by PCR and sequencing. To keep the genotyped embryos alive, a ZEG device can be used instead of using the whole embryo for the analysis [67]. For a clear example of this procedure, see [101]. **E**) To avoid phenotype variability due to mosaicism, F1 can be analyzed in addition to the injected embryos (mosaics). **F**) To produce a mutant line, founder fish are outcrossed to wild type and then F1 inbred until homozygous are found. Genotyping is performed by fin-clip or skin swabbing [129]. If the homozygote is lethal, a heterozygous line can be maintained. For an example of this procedure, see [82]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

a versatile tool was established to genetically label mutant cell clones, enabling their phenotypic analysis [47].

4.2. Knockdown versus Knockout

Since the CRISPR/Cas system has become the golden tool for studying gene function and generating genetic disease models, the use of MOs is gradually falling into disuse, and knockdown versus knockout approaches are being debated. This is due to the discrepancies found in several studies such as a study in which a screen of more than 20 zebrafish mutant lines half failed to recapitulate published MO-induced phenotypes [75]. This study also compared morphant defects published in ZFIN with the mutant phenotypes from the Sanger Zebrafish Mutation Project and found differences in approximately 80% of the phenotypes. More studies with differences in the phenotype are continuously published; for instance, disruption of *fus* using CRISPR does not induce amyotrophic lateral sclerosis (ALS) as previously reported using MOs and similar to mouse *Fus* knockout [76]. Some researchers consider that the use of MOs should be avoided or limited [77, 78]. Nevertheless, MOs are still useful for a number of purposes, particularly to complement the limitations of knockout studies. For instance, Cerebellar Atrophy with Spinal Motor Neuronopathy was correlated with variants in XOSC9 by using both CRISPR and MOs methods [79]. In loss-of-function studies, some aspects should be considered for using CRISPR or MOs and other knockdown approaches [80]: MOs can exert unnoticeable off-target effects; transcriptional adaptation to specific mutations can lead to genetic compensation [81]; mutant progeny from heterozygous parents can carry sufficient wild-type maternal mRNA to maintain normal gene function during the first stages of development; partial knockdown by MOs can result in the complete retention of normal physiological function if the targeted protein has a high affinity for its substrate or high catalytic efficiency [82]. In conclusion, both knockdown and knockout approaches are useful and complementary to elucidate the function of specific genes and understand many diseases.

5. CRISPR APPLICATIONS IN NEURAL DISEASES

Animal modeling of human brain disorders has been extensively exploited. Murine models are most commonly used to investigate the basic concepts of brain function. As previously mentioned, zebrafish have recently gained popularity as a model for studying the vertebrate brain. Below, we describe the latest applications of CRISPR/Cas9 technology for examining the brain, its fundamental structures and connectivity, and associated diseases.

5.1. Morpholinos and CRISPR/Cas9

Before the CRISPR/Cas9 system, the use of MOs in zebrafish was the most accessible and widespread way to study the (partial) loss of function of different genes involved in brain diseases or related to the structure and function of neural cells. Now, in the era of CRISPR, it is easy to generate knockout mutants to corroborate and expand the findings observed with MOs. An example of the contrasting results between MOs and CRISPRs is the outcomes of suppressing the expression of the *brf1* gene, whose mutations in humans give rise to the cerebellar-facial-skeletal syndrome. Splice blocking MOs effectively silenced the expression of both copies of the *brf1* gene (*brf1a* and *brf1b*) in zebrafish but resulted in no evident phenotypes; in contrast, CRISPR/Cas9 induced a mutation of *brf1b*, resulting in F0s at 3 days post fertilization (dpf) with symptoms similar to those presented in the human pathology. Another example is the phenotypes derived from suppressing the expression of the gene *kif15*, which codes for a member of the kinesin family. Kinesins are proteins involved in the transport of cargo along axons. MOs effectively suppress the expression of *kif15* (formerly reported as Kinesin-12) but induce extended mosaicism of the larvae, giving rise to diverse phenotypes, in particular to a different rate of growing axons with a reduced number of branches [83]. In contrast, *kif15* mutations generated by CRISPR do not present mosaicism (studied in F2 and F3); their axonal growth is accelerated, and branching is consistently reduced in embryos between 29- and 31-hours post-fertilization (hpf). Therefore, the results are more homogenous than those of MOs. In addition, the CRISPR approach also allows researchers to compare the phenotypes of homo and heterozygous mutants [84].

MOs and CRISPR-induced changes in gene expression may have different outcomes in the establishment and development of neural circuits. One case is the neural cell adhesion protein Contactin2, which is encoded by the gene *cntn2*, a glycoprotein important for neuronal migration, axon fasciculation, and the establishment of sensorimotor circuits. In this example, MOs induced axonal growth defects that are not observed when the *ctnt2* gene is removed by CRISPR. Other defects, such as delayed neuronal migration and defasciculation visualized at 48 hpf, are induced by both strategies, but it is clear that MOs may also lead to changes due to mistargeting [85].

Other studies combine observations of mutants obtained by mutagenesis screens, MOs, and CRISPR/Cas9. That is the case of the zebrafish loss-of-function model of the Transactive Response DNA-binding protein [86]. This is a model for ALS, a neurodegenerative, genetically heterogeneous disease with monogenic forms [87]. Upon comparing existing zebrafish *tardbp* mutants containing a point mutation (c660C>T) with *tardbpl* morphants and the *tardbpl* loss-of-function crispants, the point mutants did not exhibit an evident phenotype because of the compensatory role of the splice variant *tardbpl*. The splice variant *tardbpl* morphants (in the *tardbp*-/- background) and the loss-of-function *tardbpl* crispants showed severe morphological defects as early as 2 dpf, such as shorter body length and eye diameter and enlarged pericardial area, reduced life span, impaired locomotor function, decreased frequency of miniature end plate currents and perturbations in neuromuscular junction architecture. Thus, using and combining the observations of each mutant may reveal the role of specific genes and their variants in different pathologies. In a similar case, fifteen different alleles of Nodal-related protein (Ndr2) have been generated by chemical mutagenesis, radiation, and TALEN (http://www.zfin.org; *ndr2* gene information). Using CRISPR/Cas9 to target different regions in all three exons of *ndr2* gave rise to mutants at 3 dpf; these mutants showed cyclopia and phenotypes similar to those observed in hu-

mans: holoprosencephaly and heterotaxy [88]. There are two zebrafish *spastin* isoforms, ATG1 and ATG2, due to alternative translational start sites [89]. When MOs were used to independently knockdown both isoforms, fish showed two different phenotypes: 1) ATG1 mutants, which had curved tails; and 2) ATG2 mutants, which had small eyes. Both phenotypes presented locomotion defects and consequently reduced swimming speed, which appeared to be more severe in the ATG2 morphant. In contrast, CRISPR/Cas9 mutants targeting the second ATG codon prevented the synthesis of both *spastin* isoforms and disclosed its role in two separate developmental signaling pathways, one for motor circuit wiring and a second for locomotion studied larvae at 72 hpf [89]. These mutants outcrossed with fluorescent lines revealed several anatomical defects such as incorrectly developed axons. Zebrafish *shank3b* is one of the two ortholog alleles of human SHANK3, whose deficiency has been related to autism spectrum disorder (ASD). More than a dozen Shank3 mutant mice exist, but they are not suitable for use in high-throughput drug screening analysis. Previous transient zebrafish knockdowns generated by MOs for both *shank3* alleles have been reported, but developmental and behavioral studies were limited to 5 dpf [90]. Using CRISPR to genome engineer a *shank3b* loss of function mutant that was stably transmitted displayed autism-like behavioral characteristics such as impaired locomotor activity and abnormal repetitive movements, as well as impaired social preference behaviors observed at 2, 5, and 7 dpf. Shank3b deficiency caused partial lethality during early development and defective and delayed neurodevelopment in larvae. The knockout *shank3b-/-* was crossed with the transgenic line that expressed a red fluorescent protein in neurons. The fluorescent protein allowed to observe that the deficiency of this gene alters the number of neurons from the early stages of development, which was not possible to detect in mice [91]. Reduced levels of Homer1, the postsynaptic scaffolding protein, and synaptophysin, a protein located in synaptic vesicles, were also found, suggesting a potential role of Shank3 in presynaptic function [91].

5.2. Rare Diseases

Rare diseases are those that affect fewer than 1 in 2,000 people. Sometimes rare diseases are called orphan diseases because drug companies are not interested in developing drugs for their treatment. Zebrafish is a promising model for studying this kind of pathologies. One of these rare diseases is glycine encephalopathy (GE), a recessive genetic disease caused by mutations in the glycine cleavage system (glycine decarboxylase, aminomethyltransferase, hydrogen carrier protein, and dihydrolipoamide dehydrogenase). Mutations were found in the glycine decarboxylase gene in 72% of GE cases. Severe GE symptoms are heterogeneous and include severe hypotonia, myoclonic jerks, lethargy, and apnea, whereas attenuated GE symptoms are treatable seizures, spasticity, chorea, and developmental delay that can lead to intellectual disability. The zebrafish *gldc* mutants generated with CRISPR/Cas9 recapitulated GE on a molecular level and also presented a motor phenotype reminiscent of severe GE symptoms [92]. Although a transient imbalance was found in cell proliferation in the brain of the mutants, confirmed by transgenic lines expressing fluorescent reporters, the main brain networks were not affected, suggesting that GE is mainly caused by metabolic defects. Remarkably, the motor dysfunctions in the mutant larvae can be rescued by pharmacologically or genetically counterbalancing the level of glycine at the synapse, suggesting that this is a valuable model to test possible treatments for this rare disease [92]. In another case, NCAPG2 variants in two unrelated pediatric individuals with overlapping neurodevelopmental syndromic features were identified by whole-exome sequencing. NGCAP2 encodes a member of the condensin II complex, necessary for the condensation of chromosomes prior to cell division. A sole NCAPG2 ortholog was identified in the zebrafish genome (46% identity, 64% similarity). Consistent with human expression data, *ngcap2* is expressed almost ubiquitously, and predominant expression is in the developing brain and spinal cord of zebrafish larvae [93]. Injection of MOs induced a reduction in head size, whereas CRISPR/Cas9 mutants showed a significant reduction in the anterior brain. The different phenotypes can be explained because MOs have a transient knockdown effect, whereas CRISPR/Cas9 F0 crispants are considered as genetic mosaics. TUNEL and PH3 staining revealed increased cell death in both *ngcap2* morphants and CRISPR F0 mutants. Furthermore, *ngcap2* F0 mutants and morphants showed a significant increase in cell proliferation in the head [93].

Retinal function disorders have also been approached in zebrafish. Retinitis pigmentosa is a genetic disorder caused by the degeneration of rod cells. In mammals, the disease involves multiple genes including those that code for rhodopsins, essential proteins of the visual pathway. Mutations of zebrafish *rho1-1* loci induced by CRISPR/Cas offer a model for exploring disorders that mimic human diseases. The transgenic line Xops:EGFP outcrossed with rho1-1 CRISPR/Cas9 mutants showed that rod cells exhibited cell death and rhodopsin retention in the endoplasmic reticulum and Golgi apparatus but did not reach the plasma membrane [94].

5.2.1. Monogenetic Epilepsies

More examples of human pathology modeling are within the field of monogenetic epilepsies. The syntaxin-binding protein 1, STXBP1 homologs in zebrafish (Stxbp1a and Stxb1b), both homozygous mutants were characterized, which interestingly showed different features. The *stxb1a* CRISPR-induced mutant contains a 4-base pair deletion in exon 8 and is predicted to cause a premature stop codon. The *stxb1a* mutant exhibited a profound lack of movement, low electrical brain activity, low heart rate, decreased glucose and mitochondrial metabolism, and early lethality. The *stxb1b* homozygous mutant allele is a 12-base pair deletion that causes loss of the predicted start codon. The *stxb1b* mutant had spontaneous seizures and reduced locomotor activity response to a movement-inducing “dark-flash” visual stimulus, despite showing a normal metabolism, heart rate, survival, and baseline locomotor activity [95].

In 2017, two independent groups reported that the zebrafish *aldh7a1* null mutant recapitulates the characteristics of pyridoxine-dependent epilepsy (PDE) caused by variants of

the gene ALDH7A1 [82, 96]. Two homozygous mutants were generated using CRISPR/Cas9. In the first, a 5-bp deletion mutant in the zebrafish *aldh7a1* gene resulted in a premature stop codon; in the second, a 5-nucleotide insertion introduced a stop codon at position 50 of the translated protein sequence. In both studies, the mutants showed a spontaneous rapid increase in locomotion and a rapid circling swim behavior followed by seizure-like locomotor behaviors as early as 8 dpf [96] or 10 dpf [82], which resulted in death shortly after a seizure. Also, electroencephalographic recordings revealed large-amplitude spike discharges.

More recently, a report showed that the biallelic pathogenic variants of PLPBP caused a novel form of vitamin B6-dependent epilepsy. To explore the pathophysiology of the disease, the PLPHP deficiency model generated by CRISPR/Cas9 was used in a series of behavioral, biochemical, and electrophysiological studies, which showed seizure activity by 10 dpf and early death by 16 dpf. Treating the larvae with pyridoxine improved the epileptic phenotype and extended the lifespan. Thus, this model may be useful for drug discovery [97].

5.3. Brain Development

The function of the NADPH oxidase (Nox) genes of zebrafish (nox1, nox2/cybb, nox5, and duox) was addressed by inducing CRISPR/Cas9 mutations in each of the four *nox* genes. Between 48 and 96 hpf, the *nox2/cybb* chimeric crispants displayed optic nerve thinning and decreased optic tectum innervation, while the homozygous mutants showed significant ganglion cell layer expansion and mistargeted retinal axons in the optic tectum [98]. Thus, *nox* genes are relevant and have specific roles for the accurate connection between axons of retinal ganglion neurons and the optic tectum.

In other studies, it was found that the function of the *rfx4* gene, which encodes for a winged-helix transcription factor, is dispensable for forebrain morphogenesis but is required for forebrain formation. To elucidate *rfx4* function in zebrafish, two mutant alleles were made by CRISPR/Cas9, resulting in a truncated, non-functional protein [99]. Another gene implicated in neurogenesis in the neural tube is *prdm12b*. Using CRISPR/Cas9 to generate two fish lines carrying frameshift mutations in *prdm12b,* authors revealed that eng1b expression was suppressed in V1 interneurons, leading to defective Mauthner cell-dependent locomotion and embryonic lethality [100].

A customized DNA array analyzed genomic data from 66 fetuses with different brain malformations and neural tube defects and disclosed that in a sample with occipital encephalocele, an in-frame deletion in the gene that encodes for the protein WDR63 interrupted the third and fourth WD40 repeat domains. The *wdr63* gene mutation induced by CRISPR/Cas in zebrafish led to abnormal crispant embryos with body and brain malformations (40-60%) and sac-like brain protrusions (7-9%) similar to those seen in encephalocele [101]. Recently, a genome-wide linkage analysis combined with whole-genome sequencing demonstrated that human autosomal recessive primary microcephaly is caused by a mutation in the microtubule-associated protein 11 (MAP11, previously termed C7orf43). Homozygous zebrafish *map11* CRISPR/Cas9 knockout mutants recapitulated microcephaly (determined by body-head ratios) and showed decreased neuronal proliferation (determined by phosphohistone-H3 staining) [102]. Finally, a delay in retinal neurogenesis was observed upon interleukin 7 receptor (*il7r*) deletion, causing delayed myelination and revealing the regulatory role of *il7r* in this process. The homozygous mutants also showed microphthalmia with a reduced number of cones and rods and downregulation of genes, such as *rho* and *arr3a*, involved in the pathogenesis of retinitis pigmentosa [103].

5.4. Behavior

Frequently, non-stereotyped behavior reflects altered brain function due to aberrant neuronal activity. An approximation to determine the effect of specific loss-of-function genes is to select genes expressed exclusively in the brain; several examples in the literature followed this approach in zebrafish. One is the Histamine receptor H3 (Hrh3) gene knockout, a CRISPR mutant with a non-sense mutation that results in a loss of 5 to 7 transmembrane domains, which showed locomotor and social behavior impairments in larvae at 5 dpf. Hrh3 signaling regulates reactions to light:dark transitions, with *hrh3* knockout showing a faster adaptation to darkness. The mutant also has reduced levels of dopamine and serotonin, suggesting that Hrh3 is important in the modulation of other monoamine systems [104].

Another example is zebrafish *pitpnc1a*, which belongs to a family of lipid transporters of the phosphatidylinositol transfer protein (PITP). PITPs are enriched in the brain, but their functional role in neuronal signaling pathways remains elusive. The zebrafish *pitpnc1a* null mutants have a 5–base pair deletion in exon 2 that gives rise to a truncated protein lacking two key inositol binding residues. Pitpnc1 western blot does not detect the protein, indicating that the allele is functionally null. The zebrafish *pitpnc1a* null mutant is a brain-specific ortholog of the human long isoform *pitpnc1a*. By *in situ* hybridization, *pitpnc1a* mRNAs were detected in several regions of the developing central nervous system at 24 and 48 hpf. In larvae at 5 dpf, the expression was exclusively and strongly detected throughout the brain, particularly in the dorsal telencephalon. The homozygous *pitpnc1a-/-* mutant appears visually indistinguishable from wild-type siblings and is viable and fertile. However, it is also hyperactive across the day:night cycle and exhibits increased neuronal activity [105]. Interestingly, the human PITPNC1 gene resides within a copy number variant associated with a syndromic intellectual disability caused by the neighboring gene PSMD12. The CRISPR/Cas9 *psmd12* loss-of-function model exhibited microcephaly, decreased convolution of the renal tubes, and abnormal craniofacial morphology, recapitulating the human phenotypes observed in patients. F0 crispants were used to visualize the axonal tracts in the brain including the optic tecta, which were significantly smaller than in wild-type larvae at 3dpf [106]. Additional molecular characterization is needed to elucidate the implications of Psmd12 in this neurodevelopmental syndrome.

5.5. Other Applications

There is also growing interest in using zebrafish to study proteins involved in basic genome organization, dynamics, and expression, whose mutations indirectly affect the function of the nervous system. Such is the case of the studies on the function of the retinoblastoma protein RB1. The gene that codes this protein is mutated in different classes of cancer because RB1 is a tumor suppressor; thus, when the gene is mutated, the progression of the cell cycle changes, leading to tumors and cancer. RB1 mutants generated by CRISPR (heterozygous) or TALEN (genetic mosaic) in zebrafish developed tumors at 4-5 months according to the essential functional role of RB1; but this approach also allowed researchers to identify more than 170 chromatin regulators differentially expressed in RB1 tumors, including *rbbp4* and *hdac1* [36]. CRISPR/Cas heterozygous mutants of *rbbp4* are lethal between 5-10 dpf and show severe microcephaly and microphthalmia, whereas *hdac1* homozygous mutants show reduced body size, curved trunk, microcephaly, and coloboma in the retina and are lethal after 3 dpf. The requirements for *rbbp4* and *hdac1* in regulating neural progenitor proliferation and survival might contribute to oncogenesis after *rb1* loss in the zebrafish rb1 brain tumor model [36].

Zebrafish were used as a model to study how general anesthesia causes loss of consciousness after local lesioning of locus coeruleus neurons *via* two-photon laser-based ablation or genetic depletion of norepinephrine in mutants of the dopamine-beta-hydroxylase (*dbh*) gene generated by CRISPR/Cas9. Propofol and etomidate modulate firing of these neurons by inhibiting presynaptic excitatory inputs and inducing GABA-A receptor hyperpolarization in larvae between 5 and 7 dpf [107].

In other studies, from an enhancer-trap screen, an unannotated gene named *ubtor* was identified in zebrafish. *In situ* hybridization showed *ubtor* transcripts in the brain and spinal cord. To examine the function of *ubtor*, the gene was disrupted by CRISPR/Cas9 inducing higher mTOR activity and aggravated neoplasia [108]. Other experimental approximations can also be done using zebrafish and the CRISPR system, such as the characterization of potential transcriptional enhancers, like in the ZEB2 (Zinc finger E-box-binding homeobox 2) locus [109]. ZEB2 is a key developmental regulator of the central nervous system. The use of CRISPR in HEK-293 cells to delete endogenous enhancers proved that distal transcriptional enhancers and trans-acting elements govern the regulation of ZEB2 expression during neuronal development [109]. On the other hand, it is well known that endocrinological components, such as thyroid hormones, influence brain development through their roles in neurogenesis and myelination. These hormones act *via* specific receptors (THRs); however, *thr* zebrafish crispants do not show changes in expression levels of genes involved in myelination (*mbp*, *mpz*, *olig2*, and *plp*), suggesting that their function may not be directly related to the process of myelination during zebrafish early development [110].

Glial cells of zebrafish have also been studied using CRISPR/Cas as a tool. A microglia transcriptome disclosed 20 putative microglia regulators, and researchers found a dramatic decrease in neutral red-positive NR+ (nicotinamide riboside) cells when interleukin 34 (*il34*) was targeted using CRISPR/Cas9; however, microglial proliferative capacity was not affected. A stable frameshift (premature stop codon at exon 5) homozygous mutant of the *il34* receptors, *csf1a,* and *csf1b* (colony-stimulating factor 1 receptor), did not show reduced microglia numbers, suggesting a genetic compensation by other ligands [111]. A graphical overview of the genes mentioned in this section is presented in Fig. **2**. The genes and associated diseases and phenotypes mentioned in this section are summarized in Table **1**.

Despite the rapid development of genome editing technologies, specifically CRISPR/Cas9, to generate disease models in zebrafish, more efforts are needed to efficiently combine different disciplines to find both the etiology of and treatments for many brain diseases.

5.6. Improving the CRISPR System using Zebrafish

The use of CRISPR/Cas9 has been mostly restricted to knockout specific genes in zebrafish, but recent efforts have been made to expand the CRISPR toolbox in this model. For example, the injection of multiple guide RNAs to induce mutations in two different genes (*osgep* and *tprkb*) at the same time improved the efficiency of the system [112]. These genes are two of the five that code for the KEOPS complex, a series of proteins that regulate post-transcriptional modification of tRNAs, telomere length and genome maintenance, and whose mutations in humans lead to brain abnormalities and developmental delays. By knocking out the zebrafish *osgep* and *tprkb* genes with CRISPR/Cas9, homozygous mutant larvae analyzed at 4 dpf recapitulated part of the phenotype found in humans and resulted in early lethality [113].

CRISPR/Cas9 can also be used to visualize and disrupt target gene expression. Through the years there have been many reports of a number of transgenic fishes induced to express fluorescent proteins with diverse expression patterns. Important observations of brain organization and development have been revealed using these transgenic fish, which were mostly generated by non-specific incorporation of the transgene into the genome. Determining the insertion site of the transgene is laborious; however, the utility of these transgenic lines has been broadened by replacing the gene coding for the fluorescent protein by the Gal4/UAS expression system, which allows precise, inducible, and controlled gene expression [114, 115]. This gene integration strategy was performed by CRISPR/Cas9, and its applications include determining gene expression patterns to knocking in genes.

6. CRISPR AS A TOOL TO UNDERSTAND BRAIN FUNCTION

6.1. Optical Tools

The transparency of zebrafish allows the use of fluorescent proteins to monitor neural activity *in vivo*. A variety of optical tools are currently available and can be used in combination with the CRISPR/Cas system. Classically, fluorescent proteins have been used for optical screening of gene expression to determine expression patterns during development or as tools to study disease models and development

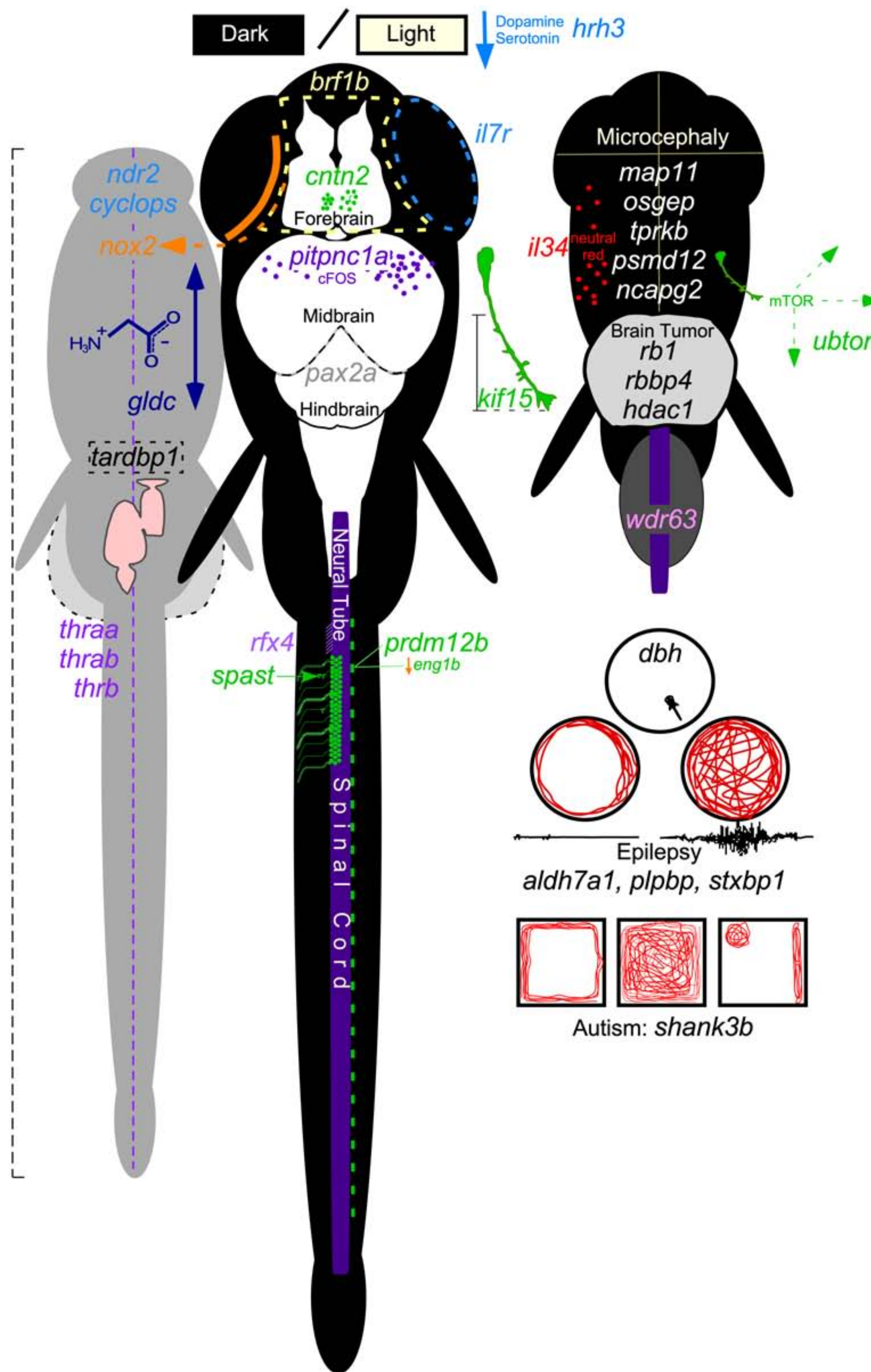

Fig. (2). Graphical representation of the genes related to neurobiology (*in italics*) edited by CRISPR/Cas to date and their associated phenotype in zebrafish. All genes are also listed in Table **1** with the corresponding reference. The main image in black illustrates the complete zebrafish (emphasizing the brain and spinal cord); genes related to the loss of midbrain-hindbrain boundaries, like *pax2a* or the optic tectum innervation (*nox2*); and genes related to neural tube formation like *rfx4*, *spast*, and *prdm12b*, among others, are included. Small zebrafish black head mainly represents the genes that produce microcephaly. A grey zebrafish shadow is a cyclops fish produced by *nrd2*/*cyclops* that includes glycine encephalopathy (*gldc*) and the morphological defects caused by disruption in *tardbp1*. Circles and squares with zebrafish larvae trajectories (in red) represent larvae motility with anesthesia (*dbh*) or the epileptic or autism phenotype. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. Gene targeted by CRISPR/Cas9, the neural disease or phenotype studied and the corresponding reference.

Gene Targeted	Disease/phenotype Studied	Refs.
aldh7a1	Pyridoxine-dependent epilepsy (PDE)	[82, 96]
brf1b	Cerebellar-facial-skeletal syndrome	[130]
cntn2	Migration (defects?) of facial branchiomotor neurons	[85]
Dbh	Genetic depletion of norepinephrine	[107]
Gldc	Glycine encephalopathy	[92]
hrh3	Responses to changes in the environment and decreased levels of dopamine and serotonin	[104]
il7r	Delay in myelination and microphthalmia	[103]
il34	Microglia number reduction	[111]
kif15	Axon regeneration	[84]
map11	Microcephaly and decreased neuronal proliferation	[102]
ncapg2	Microcephaly. Reduction in the size of the optic tecta and cerebellar hypoplasia	[93]
ndr2	Loss of the medial floor plate, severe deficits in ventral forebrain development and cyclopia	[88]
nox2	Optic nerve thinning and decreased optic tectum innervation	[98]
osgep, tprkb	Microcephaly and reduced survival	[113, 112]
pax2a	Loss of the midbrain-hindbrain boundary	[115]
pitpnc1a	Aberrant neuronal activity and increased wakefulness across the day-night cycle	[105]
plpbp	Epilepsy	[97]
prdm12b	Lack of *eng1b*-expressing V1 interneurons	[100]
psmd12	Microcephaly, decreased convolution of the renal tubes, and abnormal craniofacial morphology	[106]
rb1, rbbp4, hdac1	Brain tumors	[36]
rfx4	Role in forming midlines in the caudal neural tube	[99]
shank3b	Autism	[91]
Spast	Motor neuron and locomotion defects	[89]
stxbp1	Epilepsy. Spontaneous seizures and reduced locomotor activity	[95]
tardbp1	Morphological defects, early lethality, reduced locomotor function, aberrant quantal transmission and perturbed synapse architecture at the Neuromuscular Junctions	[86]
thraa, thrab, thrb	Loss of symmetry and laterality	[110]
Ubtor	Effects on Neurodevelopment. Cellular growth regulation and mTOR signaling	[108]
wdr63	Encephalocele and neural tube defects	[131]
zeb2	Transcriptional enhancers during neuronal development	[109]

[116]. Confocal and light-sheet microscopy made it possible to time-lapse imaging *in vivo* and track the impact of mutations on neuronal functions after injury [84].

Ionic sensors are frequently used in neurosciences to indicate neuronal activity. Genetically encoded calcium indicators (GECIs) have revolutionized the field because they can simultaneously record large numbers of neurons in the live brain [117, 118]. An example of the power of this approach is the simultaneous recording of neuronal activity in live embryos engineered with the CRISPR system to both create a transgenic fish expressing a calcium sensor under the endogenous *slc6a2* promoter (NET, the norepinephrine transporter) (Fig. **3A**), thus targeting the expression to the noradrenergic system in the brain, and monitor the activity in the *locus* coeruleus, an important region implicated in diseases like Parkinson's, Alzheimer's and autism [119]. In this

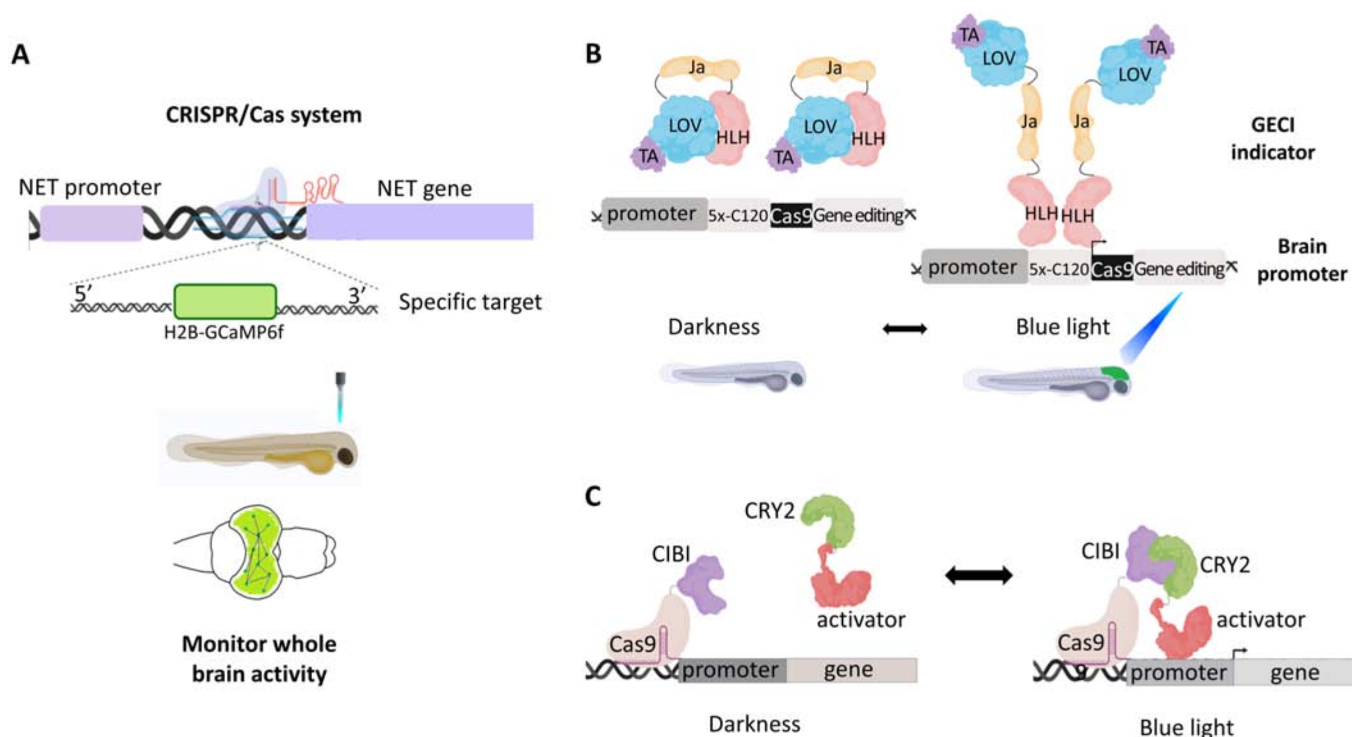

Fig. (3). CRISPR/Cas combined with optical tools in zebrafish. A) Generation of transgenic lines with CRISPR system expressing a fluorescent calcium indicator called genetically encoded calcium indicator (GECI) under promoter endogenous NET (norepinephrine transporter) to monitor noradrenergic system [119]. **B**) Optical control gene-editing protein system (TAEL). LOV (lightoxygen-voltage protein), HLH (helix-turn-helix DNA-binding domain), Ja (helix), TA photoactive transactivator (GAVPO Gal4 DNA binding domain and p65 activation domain), 5x-C120 (regulatory element termed) [122]. **C**) CRISPR/Cas combined with CRY2 light transcriptional activation. CIBI (cryptochrome interacting basic-helix-loop-helix 1), CRY2 (cryptochrome 2) [123]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

way, it is possible to monitor activity related to the development of the disease, especially when structural changes are not observed.

6.2. Optogenetic Tools

Optogenetic transcriptional activators/repressors for light-based control of transcription offer a finer spatial and temporal control of gene expression. These systems have been used in diverse animal models including zebrafish. Ideally, zebrafish optogenetic systems should be genetically encoded, reversible, not toxic and, if possible, not require complex optics. CRY2-CIB1 and EL222 have been applied to control the transcription in zebrafish embryos using the GAL4/UAS system [120]. The phytochrome B system (PHYB-PIF) has been used to modify subcellular protein localization in zebrafish embryos [121]. Recently, CRISPR/Cas9 associated with light-sensitive genes has begun to be used. In the TAEL (TA4-EL222) system, the blue-light–activated EL222 has been re-engineered, reducing toxicity but conserving fine spatial precision and rapid response kinetics. TAEL has also been combined with CRISPR/Cas9 systems to create a flexible toolkit [122] (Fig. **3B**). The advantages of using TAEL approach are that minimal changes induced by gene disruption can be detected and the expression or disruption can be limited in a specific region *in vivo* in the whole fish. CRISPR/Cas has been combined with CRY2 and its binding partner CIB1 to regulate transcriptional activation [123]. This system consists of dCas9 fusion and sgRNA; dCas9 is fused to CIB1 (trCIB1) to target a genome sequence, and the activator domain is fused to CRY2. In the absence of blue light, dCas9 recognizes genomic targets without the interaction between CIB1 and CRY2. Otherwise, CIB1 and CRY2 dimerize after exposure to blue light and sgRNAs bind to the dCas9 fusion and guide the complex to the target sites, thus activating gene expression. This system has been successfully tested for controlling the transcription level of ASCL1a, BCL6a, and HSP70 (Fig. **3C**).

7. FUTURE PERSPECTIVES AND CONCLUSION

Recent studies have used the CRISPR/Cas9 system in an attempt to confirm previous findings with MO-induced mutants and chemical mutagenesis, and also to generate new loss-of-function mutants to model neural diseases. There are still many disease-related genes to be modeled in zebrafish, not only those restricted to neural diseases. New studies using CRISPR/Cas9 will address specific functions related to structure and connectivity among brain areas. These will add to modifications and improvements of the CRISPR/Cas system and, combined with advanced optical techniques, will enable faster development of neurosciences and a better understanding of neuronal diseases. According to a recent report, now it is possible to monitor neuronal activity in freely moving zebrafish larvae using the calcium indicator GFP-aequorin. This tool can be combined with CRISPR/Cas to

eliminate some limitations of the current GECI systems. Localization, dynamics, interactions, and functions of endogenous molecules in the brain could be determined more quickly, accurately and in greater detail by combining novel imaging techniques and CRISPR transgenic zebrafish lines with fluorescent reporters.

Optogenetic tools allow the quick modification and control of many brain gene functions in vertebrates. These tools combined with the CRISPR/Cas system could help to understand neurogenerative diseases. Moreover, they have the potential of controlling and monitoring the expression of several genes at the same time, and of enhancing the understanding of complex gene networks, especially considering that many cellular responses depend on complex signaling cascades [123, 124]. Genetically encoded voltage indicators (GEVIs) have been employed to achieve fast and simultaneous detection of membrane potential in neurons. These indicators report fast activity in correlation with action potential and present high brightness and less cellular damage compared to GECIs, thus improving neural activity recordings [125]. However, they have been used in a few physiological conditions [126-128]. The CRISPR/Cas system could facilitate the use of GEVIs in zebrafish to characterize functional connectomes *in vivo*, allowing researchers to understand neurological processes and diseases.

CRISPR is routinely used to generate knockouts in specific genes. Interestingly, CRISPR can generate knockins and precise genome editions, which will enable scientists to reproduce the mutations equivalent to those in patients to set up personalized medicine. Genome editing with the CRISPR system and derived technologies will undoubtedly accelerate the development of neurosciences using zebrafish and other animal models to finally translate to benefit humans.

LIST OF ABBREVIATIONS

ALS = Amyotrophic lateral sclerosis

ASD = Autism spectrum disorder

CIBI = Cryptochrome interacting basic helix-loop helix 1

CRISPR/Cas = Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein

CRY2 = Cryptochrome 2

Dbh = Dopamine-beta-hydroxylase

DSB = Double-strand breaks

dpf = Days post fertilization

eGFP = Enhanced green fluorescent protein

GABA = γ-Aminobutyric acid

GE = Glycine encephalopathy

GECIs = Genetically encoded calcium indicators

GEVIs = Genetically encoded voltage indicators

HMA = Heteroduplex mobility assay

HSP = Cause hereditary spastic paraplegia

InDels = Insertions and/or deletions

MOs = Morpholinos

NET = Norepinephrine transporter

NHEJ = Non-homologous end-joining

Nox = NADPH oxidase

NR = Nicotinamide riboside

ORF = Open reading frame

PDE = Pyridoxine-dependent epilepsy

PHYB-PIF = Phytochrome B system

PITP = Phosphatidylinositol transfer protein

sgRNA = Single-guide RNA

TAEL = (TA4-EL222) system

TALENs = Transcription activator-like effector nucleases

TIDE = Tracking of indels by decomposition

ViBE-Z = Virtual brain explorer for zebrafish

ZEB2 = Zinc finger E-box-binding homeobox 2

ZFNs = Zinc finger nucleases

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

The study was supported by grants from CONACYT to AMT (A1-S-7659) and to LRRO (A1-S-10450).

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors thank LCC Jessica González Norris for proofreading the English version of this manuscript.

REFERENCES

[1] Walker, C.; Streisinger, G. Induction of mutations by gamma-rays in pregonial germ cells of Zebrafish embryos. *Genetics,* **1983**, *103*(1), 125-136. [PMID: 17246099]

[2] Kimmel, C.B.; Ballard, W.W.; Kimmel, S.R.; Ullmann, B.; Schilling, T.F. Stages of embryonic development of the zebrafish. *Dev. Dyn.,* **1995**, *203*(3), 253-310. [http://dx.doi.org/10.1002/aja.1002030302] [PMID: 8589427]

[3] Dorsemans, A-C.; Soulé, S.; Weger, M.; Bourdon, E.; Lefebvre d'Hellencourt, C.; Meilhac, O.; Diotel, N. Impaired constitutive and regenerative neurogenesis in adult hyperglycemic zebrafish. *J. Comp. Neurol.,* **2017**, *525*(3), 442-458. [http://dx.doi.org/10.1002/cne.24065] [PMID: 27339277]

[4] Varshney, G.K.; Sood, R.; Burgess, S.M. Understanding and Editing the Zebrafish Genome. *Adv. Genet.,* **2015**, *92*, 1-52. [http://dx.doi.org/10.1016/bs.adgen.2015.09.002] [PMID: 26639914]

[5] Mueller, T.; Wullimann, M.F.; Guo, S. Early teleostean basal ganglia development visualized by zebrafish Dlx2a, Lhx6, Lhx7, Tbr2 (eomesa), and GAD67 gene expression. *J. Comp. Neurol.,* **2008**, *507*(2), 1245-1257. [http://dx.doi.org/10.1002/cne.21604] [PMID: 18181142]

[6] Schmidt, R.; Strähle, U.; Scholpp, S.; Altman, J.; Das, G.; Altman, J.; Lois, C.; Alvarez-Buylla, A.; Gage, F.; Coates, P. Neurogenesis in zebrafish - from embryo to adult. *Neural Dev.,* **2013**, *8*(1), 3. [http://dx.doi.org/10.1186/1749-8104-8-3] [PMID: 23433260]

[7] Randlett, O.; Wee, C.L.; Naumann, E.A.; Nnaemeka, O.; Schoppik, D.; Fitzgerald, J.E.; Portugues, R.; Lacoste, A.M.B.; Riegler, C.; Engert, F.; Schier, A.F. Whole-brain activity mapping onto a zebrafish brain atlas. *Nat. Methods,* **2015**, *12*(11), 1039-1046. [http://dx.doi.org/10.1038/nmeth.3581] [PMID: 26778924]

[8] Hildebrand, D.G.C.; Cicconet, M.; Torres, R.M.; Choi, W.; Quan, T.M.; Moon, J.; Wetzel, A.W.; Scott Champion, A.; Graham, B.J.; Randlett, O.; Plummer, G.S.; Portugues, R.; Bianco, I.H.; Saalfeld, S.; Baden, A.D.; Lillaney, K.; Burns, R.; Vogelstein, J.T.; Schier, A.F.; Lee, W.A.; Jeong, W.K.; Lichtman, J.W.; Engert, F. Whole-brain serial-section electron microscopy in larval zebrafish. *Nature,* **2017**, *545*(7654), 345-349. [http://dx.doi.org/10.1038/nature22356] [PMID: 28489821]

[9] Cordero-maldonado, M.L.; Perathoner, S.; Van Der Kolk, K.; Boland, R.; Heins-marroquin, U.; Spaink, H.P.; Meijer, A.H.; Crawford, A.D.; De Sonneville, J.; Nauheim, B. Deep learning image recognition enables efficient genome editing in Zebrafish by automated injections*;* No. Mlcm, **2018**, pp. 1-18.

[10] Perathoner, S.; Cordero-Maldonado, M.L.; Crawford, A.D. Potential of zebrafish as a model for exploring the role of the amygdala in emotional memory and motivational behavior. *J. Neurosci. Res.,* **2016**, *94*(6), 445-462. [http://dx.doi.org/10.1002/jnr.23712] [PMID: 26833658]

[11] Blader, P.; Strähle, U. Zebrafish developmental genetics and central nervous system development. *Hum. Mol. Genet.,* **2000**, *9*(6), 945-951. [http://dx.doi.org/10.1093/hmg/9.6.945] [PMID: 10767318]

[12] Ota, S.; Kawahara, A. Zebrafish: a model vertebrate suitable for the analysis of human genetic disorders. *Congenit. Anom. (Kyoto),* **2014**, *54*(1), 8-11. [http://dx.doi.org/10.1111/cga.12040] [PMID: 24279334]

[13] Sakai, C.; Ijaz, S.; Hoffman, E.J. Zebrafish models of neurodevelopmental disorders: Past, present, and future. *Front. Mol. Neurosci.,* **2018**, *11*, 294. [http://dx.doi.org/10.3389/fnmol.2018.00294] [PMID: 30210288]

[14] Fontana, B.D.; Mezzomo, N.J.; Kalueff, A.V.; Rosemberg, D.B. The developing utility of zebrafish models of neurological and neuropsychiatric disorders: A critical review. *Exp. Neurol.,* **2018**, *299*(Pt A), 157-171. [http://dx.doi.org/10.1016/j.expneurol.2017.10.004] [PMID: 28987462]

[15] Kalueff, A.V.; Stewart, A.M.; Gerlai, R. Zebrafish as an emerging model for studying complex brain disorders. *Trends Pharmacol. Sci.,* **2014**, *35*(2), 63-75. [http://dx.doi.org/10.1016/j.tips.2013.12.002] [PMID: 24412421]

[16] Wiley, D.S.; Redfield, S.E.; Zon, L.I. Chemical screening in zebrafish for novel biological and therapeutic discovery. *Methods Cell Biol.,* **2017**, *138*, 651-679. [http://dx.doi.org/10.1016/bs.mcb.2016.10.004] [PMID: 28129862]

[17] MacRae, C.A.; Peterson, R.T. Zebrafish as tools for drug discovery. *Nat. Rev. Drug Discov.,* **2015**, *14*(10), 721-731. [http://dx.doi.org/10.1038/nrd4627] [PMID: 26361349]

[18] Avey, M.T.; Fenwick, N.; Griffin, G. The use of systematic reviews and reporting guidelines to advance the implementation of the 3Rs. *J. Am. Assoc. Lab. Anim. Sci.,* **2015**, *54*(2), 153-162. [PMID: 25836961]

[19] Howe, K.; Clark, M.D.; Torroja, C.F.; Torrance, J.; Berthelot, C.; Muffato, M.; Collins, J.E.; Humphray, S.; McLaren, K.; Matthews, L.; McLaren, S.; Sealy, I.; Caccamo, M.; Churcher, C.; Scott, C.; Barrett, J.C.; Koch, R.; Rauch, G.J.; White, S.; Chow, W.; Kilian, B.; Quintais, L.T.; Guerra-Assunção, J.A.; Zhou, Y.; Gu, Y.; Yen, J.; Vogel, J.H.; Eyre, T.; Redmond, S.; Banerjee, R.; Chi, J.; Fu, B.; Langley, E.; Maguire, S.F.; Laird, G.K.; Lloyd, D.; Kenyon, E.; Donaldson, S.; Sehra, H.; Almeida-King, J.; Loveland, J.; Trevanion, S.; Jones, M.; Quail, M.; Willey, D.; Hunt, A.; Burton, J.; Sims, S.; McLay, K.; Plumb, B.; Davis, J.; Clee, C.; Oliver, K.; Clark, R.; Riddle, C.; Elliot, D.; Threadgold, G.; Harden, G.; Ware, D.; Begum, S.; Mortimore, B.; Kerry, G.; Heath, P.; Phillimore, B.; Tracey, A.; Corby, N.; Dunn, M.; Johnson, C.; Wood, J.; Clark, S.; Pelan, S.; Griffiths, G.; Smith, M.; Glithero, R.; Howden, P.; Barker, N.; Lloyd, C.; Stevens, C.; Harley, J.; Holt, K.; Panagiotidis, G.; Lovell, J.; Beasley, H.; Henderson, C.; Gordon, D.; Auger, K.; Wright, D.; Collins, J.; Raisen, C.; Dyer, L.; Leung, K.; Robertson, L.; Ambridge, K.; Leongamornlert, D.; McGuire, S.; Gilderthorp, R.; Griffiths, C.; Manthravadi, D.; Nichol, S.; Barker, G.; Whitehead, S.; Kay, M.; Brown, J.; Murnane, C.; Gray, E.; Humphries, M.; Sycamore, N.; Barker, D.; Saunders, D.; Wallis, J.; Babbage, A.; Hammond, S.; Mashreghi-Mohammadi, M.; Barr, L.; Martin, S.; Wray, P.; Ellington, A.; Matthews, N.; Ellwood, M.; Woodmansey, R.; Clark, G.; Cooper, J.; Tromans, A.; Grafham, D.; Skuce, C.; Pandian, R.; Andrews, R.; Harrison, E.; Kimberley, A.; Garnett, J.; Fosker, N.; Hall, R.; Garner, P.; Kelly, D.; Bird, C.; Palmer, S.; Gehring, I.; Berger, A.; Dooley, C.M.; Ersan-Ürün, Z.; Eser, C.; Geiger, H.; Geisler, M.; Karotki, L.; Kirn, A.; Konantz, J.; Konantz, M.; Oberländer, M.; Rudolph-Geiger, S.; Teucke, M.; Lanz, C.; Raddatz, G.; Osoegawa, K.; Zhu, B.; Rapp, A.; Widaa, S.; Langford, C.; Yang, F.; Schuster, S.C.; Carter, N.P.; Harrow, J.; Ning, Z.; Herrero, J.; Searle, S.M.; Enright, A.; Geisler, R.; Plasterk, R.H.; Lee, C.; Westerfield, M.; de Jong, P.J.; Zon, L.I.; Postlethwait, J.H.; Nüsslein-Volhard, C.; Hubbard, T.J.; Roest Crollius, H.; Rogers, J.; Stemple, D.L. The zebrafish reference genome sequence and its relationship to the human genome. *Nature,* **2013**, *496*(7446), 498-503. [http://dx.doi.org/10.1038/nature12111] [PMID: 23594743]

[20] Lambert, M.J.; Cochran, W.O.; Wilde, B.M.; Olsen, K.G.; Cooper, C.D. Evidence for widespread subfunctionalization of splice forms in vertebrate genomes. *Genome Res.,* **2015**, *25*(5), 624-632. [http://dx.doi.org/10.1101/gr.184473.114] [PMID: 25792610]

[21] Haffter, P.; Granato, M.; Brand, M.; Mullins, M.C.; Hammerschmidt, M.; Kane, D.A.; Odenthal, J.; van Eeden, F.J.; Jiang, Y.J.; Heisenberg, C.P.; Kelsh, R.N.; Furutani-Seiki, M.; Vogelsang, E.; Beuchle, D.; Schach, U.; Fabian, C.; Nüsslein-Volhard, C. The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development,* **1996**, *123*, 1-36. [PMID: 9007226]

[22] Lin, S.; Gaiano, N.; Culp, P.; Burns, J.C.; Friedmann, T.; Yee, J.K.; Hopkins, N. Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish. *Science,* **1994**, *265*(5172), 666-669. [http://dx.doi.org/10.1126/science.8036514] [PMID: 8036514]

[23] Amsterdam, A.; Burgess, S.; Golling, G.; Chen, W.; Sun, Z.; Townsend, K.; Farrington, S.; Haldi, M.; Hopkins, N. A large-scale insertional mutagenesis screen in zebrafish. *Genes Dev.,* **1999**, *13*(20), 2713-2724. [http://dx.doi.org/10.1101/gad.13.20.2713] [PMID: 10541557]

[24] Golling, G.; Amsterdam, A.; Sun, Z.; Antonelli, M.; Maldonado, E.; Chen, W.; Burgess, S.; Haldi, M.; Artzt, K.; Farrington, S.; Lin, S.Y.; Nissen, R.M.; Hopkins, N. Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development. *Nat. Genet.,* **2002**, *31*(2), 135-140. [http://dx.doi.org/10.1038/ng896] [PMID: 12006978]

[25] Kawakami, K.; Koga, A.; Hori, H.; Shima, A. Excision of the tol2 transposable element of the medaka fish, *Oryzias latipes*, in zebrafish, *Danio rerio*. *Gene,* **1998**, *225*(1-2), 17-22. [http://dx.doi.org/10.1016/S0378-1119(98)00537-X] [PMID: 9931412]

[26] Davidson, A.E.; Balciunas, D.; Mohn, D.; Shaffer, J.; Hermanson, S.; Sivasubbu, S.; Cliff, M.P.; Hackett, P.B.; Ekker, S.C. Efficient gene delivery and gene expression in zebrafish using the sleeping beauty transposon. *Dev. Biol.,* **2003**, *263*(2), 191-202. [http://dx.doi.org/10.1016/j.ydbio.2003.07.013] [PMID: 14597195]

[27] Emelyanov, A.; Gao, Y.; Naqvi, N.I.; Parinov, S. Trans-kingdom transposition of the maize dissociation element. *Genetics,* **2006**, *174*(3), 1095-1104. [http://dx.doi.org/10.1534/genetics.106.061184] [PMID: 16951067]

[28] Bergamin, G.; Cieri, D.; Vazza, G.; Argenton, F.; Mostacciuolo, M.L.; Argenton, F. Zebrafish Tg(hb9:MTS-Kaede): a new *in vivo* tool for studying the axonal movement of mitochondria. *Biochim. Biophys. Acta,* **2016**, *1860*(6), 1247-1255. [http://dx.doi.org/10.1016/j.bbagen.2016.03.007] [PMID: 26968460]

[29] Ni, J.; Wangensteen, K.J.; Nelsen, D.; Balciunas, D.; Skuster, K.J.; Urban, M.D.; Ekker, S.C. Active recombinant Tol2 transposase for gene transfer and gene discovery applications. *Mob. DNA,* **2016**, *7*, 6.

[http://dx.doi.org/10.1186/s13100-016-0062-z] [PMID: 27042235]

[30] Wheeler, M.A.; Smith, C.J.; Ottolini, M.; Barker, B.S.; Purohit, A.M.; Grippo, R.M.; Gaykema, R.P.; Spano, A.J.; Beenhakker, M.P.; Kucenas, S.; Patel, M.K.; Deppmann, C.D.; Güler, A.D. Genetically targeted magnetic control of the nervous system. *Nat. Neurosci.,* **2016**, *19*(5), 756-761. [http://dx.doi.org/10.1038/nn.4265] [PMID: 26950006]

[31] Doyon, Y.; McCammon, J.M.; Miller, J.C.; Faraji, F.; Ngo, C.; Katibah, G.E.; Amora, R.; Hocking, T.D.; Zhang, L.; Rebar, E.J.; Gregory, P.D.; Urnov, F.D.; Amacher, S.L. Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nat. Biotechnol.,* **2008**, *26*(6), 702-708. [http://dx.doi.org/10.1038/nbt1409] [PMID: 18500334]

[32] Meng, X.; Noyes, M.B.; Zhu, L.J.; Lawson, N.D.; Wolfe, S.A. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat. Biotechnol.,* **2008**, *26*(6), 695-701. [http://dx.doi.org/10.1038/nbt1398] [PMID: 18500337]

[33] Huang, P.; Xiao, A.; Zhou, M.; Zhu, Z.; Lin, S.; Zhang, B. Heritable gene targeting in zebrafish using customized TALENs. *Nat. Biotechnol.,* **2011**, *29*(8), 699-700. [http://dx.doi.org/10.1038/nbt.1939] [PMID: 21822242]

[34] Bedell, V.M.; Wang, Y.; Campbell, J.M.; Poshusta, T.L.; Starker, C.G.; Krug, R.G., II; Tan, W.; Penheiter, S.G.; Ma, A.C.; Leung, A.Y.H.; Fahrenkrug, S.C.; Carlson, D.F.; Voytas, D.F.; Clark, K.J.; Essner, J.J.; Ekker, S.C. *In vivo* genome editing using a high-efficiency TALEN system. *Nature,* **2012**, *491*(7422), 114-118. [http://dx.doi.org/10.1038/nature11537] [PMID: 23000899]

[35] Kise, R.; Okasato, R.; Kano, K.; Inoue, A.; Kawahara, A.; Aoki, J. Identification and biochemical characterization of a second zebrafish autotaxin gene. *J. Biochem.,* **2019**, *165*(3), 269-275. [http://dx.doi.org/10.1093/jb/mvy114] [PMID: 30629186]

[36] Schultz, L.E.; Haltom, J.A.; Almeida, M.P.; Wierson, W.A.; Solin, S.L.; Weiss, T.J.; Helmer, J.A.; Sandquist, E.J.; Shive, H.R.; McGrail, M. Epigenetic regulators Rbbp4 and Hdac1 are overexpressed in a zebrafish model of RB1 embryonal brain tumor, and are required for neural progenitor survival and proliferation. *Dis. Model. Mech.,* **2018**, *11*(6), dmm034124. [http://dx.doi.org/10.1242/dmm.034124] [PMID: 29914980]

[37] Audira, G.; Sarasamma, S.; Chen, J-R.; Juniardi, S.; Sampurna, B.P.; Liang, S-T.; Lai, Y-H.; Lin, G-M.; Hsieh, M-C.; Hsiao, C-D. Zebrafish Mutants Carrying Leptin a (lepa) Gene Deficiency Display Obesity, Anxiety, Less Aggression and Fear, and Circadian Rhythm and Color Preference Dysregulation. *Int. J. Mol. Sci.,* **2018**, *19*(12), 4038. [http://dx.doi.org/10.3390/ijms19124038] [PMID: 30551684]

[38] Nasevicius, A.; Ekker, S.C. Effective targeted gene ‘knockdown’ in zebrafish. *Nat. Genet.,* **2000**, *26*(2), 216-220. [http://dx.doi.org/10.1038/79951] [PMID: 11017081]

[39] Flynt, A.S.; Rao, M.; Patton, J.G. Blocking Zebrafish MicroRNAs with Morpholinos. *Methods Mol. Biol.,* **2017**, *1565*, 59-78. [http://dx.doi.org/10.1007/978-1-4939-6817-6_6] [PMID: 28364234]

[40] De Rienzo, G.; Gutzman, J.H.; Sive, H. Efficient shRNA-mediated inhibition of gene expression in zebrafish. *Zebrafish,* **2012**, *9*(3), 97-107. [http://dx.doi.org/10.1089/zeb.2012.0770] [PMID: 22788660]

[41] Dong, Z.; Peng, J.; Guo, S. Stable gene silencing in zebrafish with spatiotemporally targetable RNA interference. *Genetics,* **2013**, *193*(4), 1065-1071. [http://dx.doi.org/10.1534/genetics.112.147892] [PMID: 23378068]

[42] Andrews, O.E.; Cha, D.J.; Wei, C.; Patton, J.G. RNAi-mediated gene silencing in zebrafish triggered by convergent transcription. *Sci. Rep.,* **2014**, *4*, 5222. [http://dx.doi.org/10.1038/srep05222] [PMID: 24909225]

[43] Marraffini, L.A.; Sontheimer, E.J. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat. Rev. Genet.,* **2010**, *11*(3), 181-190. [http://dx.doi.org/10.1038/nrg2749] [PMID: 20125085]

[44] Hwang, W.Y.; Fu, Y.; Reyon, D.; Maeder, M.L.; Tsai, S.Q.; Sander, J.D.; Peterson, R.T.; Yeh, J-R.J.; Joung, J.K. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat. Biotechnol.,* **2013**, *31*(3), 227-229. [http://dx.doi.org/10.1038/nbt.2501] [PMID: 23360964]

[45] Jao, L-E.; Wente, S.R.; Chen, W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc. Natl. Acad. Sci. USA,* **2013**, *110*(34), 13904-13909. [http://dx.doi.org/10.1073/pnas.1308335110] [PMID: 23918387]

[46] Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J. A.; Charpentier, E. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science,* **2012**, *337*, 6096. [http://dx.doi.org/10.1126/science.1225829]

[47] Di Donato, V.; De Santis, F.; Auer, T.O.; Testa, N.; Sánchez-Iranzo, H.; Mercader, N.; Concordet, J-P.; Del Bene, F. 2C-Cas9: a versatile tool for clonal analysis of gene function. *Genome Res.,* **2016**, *26*(5), 681-692. [http://dx.doi.org/10.1101/gr.196170.115] [PMID: 26957310]

[48] Cho, S.W.; Kim, S.; Kim, J.M.; Kim, J-S. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. *Nat. Biotechnol.,* **2013**, *31*(3), 230-232. [http://dx.doi.org/10.1038/nbt.2507] [PMID: 23360966]

[49] Kleinstiver, B.P.; Prew, M.S.; Tsai, S.Q.; Topkar, V.V.; Nguyen, N.T.; Zheng, Z.; Gonzales, A.P.W.; Li, Z.; Peterson, R.T.; Yeh, J-R.J.; Aryee, M.J.; Joung, J.K. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature,* **2015**, *523*(7561), 481-485. [http://dx.doi.org/10.1038/nature14592] [PMID: 26098369]

[50] Xu, X.; Qi, L.S. A CRISPR-dCas Toolbox for Genetic Engineering and Synthetic Biology. *J. Mol. Biol.,* **2019**, *431*(1), 34-47. [http://dx.doi.org/10.1016/j.jmb.2018.06.037] [PMID: 29958882]

[51] Wu, W.; Yang, Y.; Lei, H. Progress in the application of CRISPR: From gene to base editing. *Med. Res. Rev.,* **2019**, *39*(2), 665-683. [http://dx.doi.org/10.1002/med.21537] [PMID: 30171624]

[52] Cai, M.; Han, L.; Liu, L.; He, F.; Chu, W.; Zhang, J.; Tian, Z.; Du, S. *Defective Sarcomere Assembly in Smyd1a and Smyd1b Zebrafish Mutants., FASEB J.,* **2019**, fj.201801578R. [http://dx.doi.org/10.1096/fj.201801578R]

[53] Varshney, G. K.; Pei, W.; Lafave, M. C.; Idol, J.; Xu, L.; Gallardo, V.; Carrington, B.; Bishop, K.; Jones, M.; Li, M. *High-Throughput Gene Targeting and Phenotyping in Zebrafish Using CRISPR / Cas9,* **2015**, 1030–1042. [http://dx.doi.org/10.1101/gr.186379.114.Freely]

[54] Liu, D.; Wang, Z.; Xiao, A.; Zhang, Y.; Li, W.; Zu, Y.; Yao, S.; Lin, S.; Zhang, B. Efficient gene targeting in zebrafish mediated by a zebrafish-codon-optimized cas9 and evaluation of off-targeting effect. *J. Genet. Genomics,* **2014**, *41*(1), 43-46. [http://dx.doi.org/10.1016/j.jgg.2013.11.004] [PMID: 24480746]

[55] Shiraki, T.; Kawakami, K. A tRNA-based multiplex sgRNA expression system in zebrafish and its application to generation of transgenic albino fish. *Sci. Rep.,* **2018**, *8*(1), 13366. [http://dx.doi.org/10.1038/s41598-018-31476-5] [PMID: 30190522]

[56] Zhang, Y.; Zhang, Z.; Ge, W. An efficient platform for generating somatic point mutations with germline transmission in the zebrafish by CRISPR/Cas9-mediated gene editing. *J. Biol. Chem*., **2018**, *293*(17), 6611-6622.

[57] Prykhozhij, S.V.; Fuller, C.; Steele, S.L.; Veinotte, C.J.; Razaghi, B.; Robitaille, J.M.; McMaster, C.R.; Shlien, A.; Malkin, D.; Berman, J.N. Optimized knock-in of point mutations in zebrafish using CRISPR/Cas9. *Nucleic Acids Res.,* **2018**, *46*(17), e102. [http://dx.doi.org/10.1093/nar/gky512] [PMID: 29905858]

[58] Fernandez, J.P.; Vejnar, C.E.; Giraldez, A.J.; Rouet, R.; Moreno-Mateos, M.A. Optimized CRISPR-Cpf1 system for genome editing in zebrafish. *Methods,* **2018**, *150*(March), 11-18. [http://dx.doi.org/10.1016/j.ymeth.2018.06.014] [PMID: 29964176]

[59] Labun, K.; Montague, T.G.; Gagnon, J.A.; Thyme, S.B.; Valen, E. CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. *Nucleic Acids Res.,* **2016**, *44*(W1), W272-6. [http://dx.doi.org/10.1093/nar/gkw398] [PMID: 27185894]

[60] Moreno-Mateos, M.A.; Vejnar, C.E.; Beaudoin, J.D.; Fernandez, J.P.; Mis, E.K.; Khokha, M.K.; Giraldez, A.J. CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting *in vivo*. *Nat. Methods,* **2015**, *12*(10), 982-988. [http://dx.doi.org/10.1038/nmeth.3543] [PMID: 26322839]

[61] Gagnon, J.A.; Valen, E.; Thyme, S.B.; Huang, P.; Akhmetova, L.; Pauli, A.; Montague, T.G.; Zimmerman, S.; Richter, C.; Schier, A.F. Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. *PLoS One,* **2014**, *9*(5), e98186. [http://dx.doi.org/10.1371/journal.pone.0098186] [PMID: 24873830]

[62] Zhao, Y.; Sun, H.; Sha, X.; Gu, L.; Zhan, Z.; Li, W.; Zhao, Y.; Sun, H.; Sha, X.; Gu, L. A Review of Automated Microinjection of Zebrafish Embryos. *Micromachines [Internet].,* **2018**, *10*(1), 7. Http://Www.Mdpi.Com/2072-666X/10/1/7ew.

[http://dx.doi.org/10.3390/mi10010007]

[63] Pei, W.; Burgess, S.M. Microinjection in Zebrafish for Genome Editing and Functional Studies. *Methods Mol. Biol.,* **2019**, *1874*, 459-474. [http://dx.doi.org/10.1007/978-1-4939-8831-0_26] [PMID: 30353530]

[64] D'Agostino, Y.; Locascio, A.; Ristoratore, F.; Sordino, P.; Spagnuolo, A.; Borra, M.; D'Aniello, S. A rapid and cheap methodology for CRISPR/Cas9 zebrafish mutant screening. *Mol. Biotechnol*., **2015**, *58*(1), 73-8.

[65] Foster, S.D.; Glover, S.R.; Turner, A.N.; Chatti, K.; Challa, A.K. A mixing heteroduplex mobility assay (mHMA) to genotype homozygous mutants with small indels generated by CRISPR-Cas9 nucleases. *MethodsX,* **2018**, *6*, 1-5. [http://dx.doi.org/10.1016/j.mex.2018.11.017] [PMID: 30591915]

[66] Brinkman, E.K.; Chen, T.; Amendola, M.; van Steensel, B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res.,* **2014**, *42*(22), e168-e168. [http://dx.doi.org/10.1093/nar/gku936] [PMID: 25300484]

[67] Lambert, C.J.; Freshner, B.C.; Chung, A.; Stevenson, T.J.; Bowles, D.M.; Samuel, R.; Gale, B.K.; Bonkowsky, J.L. An automated system for rapid cellular extraction from live zebrafish embryos and larvae: Development and application to genotyping. *PLoS One,* **2018**, *13*(3), e0193180. [http://dx.doi.org/10.1371/journal.pone.0193180] [PMID: 29543903]

[68] Vejnar, C. E.; Moreno-Mateos, M. A.; Cifuentes, D.; Bazzini, A. A.; Giraldez, A. J. Optimized CRISPR-Cas9 system for genome editing in Zebrafish. *Cold Spring Harb. Protoc.,* **2016**, *10*, pdb.prot086850. [http://dx.doi.org/10.1101/pdb.prot086850]

[69] Rafferty, S.A.; Quinn, T.A. A beginner's guide to understanding and implementing the genetic modification of zebrafish. *Prog. Biophys. Mol. Biol.,* **2018**, *138*, 3-19. [http://dx.doi.org/10.1016/j.pbiomolbio.2018.07.005] [PMID: 30032905]

[70] Yang, Z.; Chen, S.; Xue, S.; Li, X.; Sun, Z.; Yang, Y.; Hu, X.; Geng, T.; Cui, H. Generation of Cas9 transgenic zebrafish and their application in establishing an ERV-deficient animal model. *Biotechnol. Lett.,* **2018**, *40*(11-12), 1507-1518. [http://dx.doi.org/10.1007/s10529-018-2605-5] [PMID: 30244429]

[71] Li, Jia; Zhang, Baibing; Bu, Jiwen; Du, J. Intron-based genomic editing: A highly efficient method for generating knockin zebrafish. *Oncotarget*, **2015**, *6*(20), 17891-4. [http://dx.doi.org/10.18632/oncotarget.4547]

[72] Hisano, Y.; Sakuma, T.; Nakade, S.; Ohga, R.; Ota, S.; Okamoto, H.; Yamamoto, T.; Kawahara, A. Precise in-frame integration of exogenous DNA mediated by CRISPR/Cas9 system in zebrafish. *Sci. Rep.,* **2015**, *5*, 8841. [http://dx.doi.org/10.1038/srep08841] [PMID: 25740433]

[73] Simone, B.W.; Martínez-Gálvez, G.; WareJoncas, Z.; Ekker, S.C. Fishing for understanding: Unlocking the zebrafish gene editor's toolbox. *Methods,* **2018**, *150*(July), 3-10. [http://dx.doi.org/10.1016/j.ymeth.2018.07.012] [PMID: 30076892]

[74] Albadri, S.; Del Bene, F.; Revenu, C. Genome editing using CRISPR/Cas9-based knock-in approaches in zebrafish. *Methods,* **2017**, *121-122*, 77-85. [http://dx.doi.org/10.1016/j.ymeth.2017.03.005] [PMID: 28300641]

[75] Kok, F.O.; Shin, M.; Ni, C-W.; Gupta, A.; Grosse, A.S.; van Impel, A.; Kirchmaier, B.C.; Peterson-Maduro, J.; Kourkoulis, G.; Male, I.; DeSantis, D.F.; Sheppard-Tindell, S.; Ebarasi, L.; Betsholtz, C.; Schulte-Merker, S.; Wolfe, S.A.; Lawson, N.D. Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. *Dev. Cell,* **2015**, *32*(1), 97-108. [http://dx.doi.org/10.1016/j.devcel.2014.11.018] [PMID: 25533206]

[76] Lebedeva, S.; de Jesus Domingues, A.M.; Butter, F.; Ketting, R.F. Characterization of genetic loss-of-function of Fus in zebrafish. *RNA Biol.,* **2017**, *14*(1), 29-35. [http://dx.doi.org/10.1080/15476286.2016.1256532] [PMID: 27898262]

[77] Stainier, D.Y.R.; Raz, E.; Lawson, N.D.; Ekker, S.C.; Burdine, R.D.; Eisen, J.S.; Ingham, P.W.; Schulte-Merker, S.; Yelon, D.; Weinstein, B.M.; Mullins, M.C.; Wilson, S.W.; Ramakrishnan, L.; Amacher, S.L.; Neuhauss, S.C.F.; Meng, A.; Mochizuki, N.; Panula, P.; Moens, C.B. Guidelines for morpholino use in zebrafish. *PLoS Genet.,* **2017**, *13*(10), e1007000. [http://dx.doi.org/10.1371/journal.pgen.1007000] [PMID: 29049395]

[78] Lawson, N.D. Reverse genetics in Zebrafish: Mutants, morphants, and moving forward. *Trends Cell Biol.,* **2016**, *26*(2), 77-79. [http://dx.doi.org/10.1016/j.tcb.2015.11.005] [PMID: 26739910]

[79] Burns, D.T.; Donkervoort, S.; Müller, J.S.; Knierim, E.; Bharucha-Goebel, D.; Faqeih, E.A.; Bell, S.K.; AlFaifi, A.Y.; Monies, D.; Millan, F.; Retterer, K.; Dyack, S.; MacKay, S.; Morales-Gonzalez, S.; Giunta, M.; Munro, B.; Hudson, G.; Scavina, M.; Baker, L.; Massini, T.C.; Lek, M.; Hu, Y.; Ezzo, D.; AlKuraya, F.S.; Kang, P.B.; Griffin, H.; Foley, A.R.; Schuelke, M.; Horvath, R.; Bönnemann, C.G. Variants in EXOSC9 Disrupt the RNA Exosome and Result in Cerebellar Atrophy with Spinal Motor Neuronopathy. *Am. J. Hum. Genet.,* **2018**, *102*(5), 858-873. [http://dx.doi.org/10.1016/j.ajhg.2018.03.011] [PMID: 29727687]

[80] Zimmer, A.M.; Pan, Y.K.; Chandrapalan, T.; Kwong, R.W.M.; Perry, S.F. Loss-of-function approaches in comparative physiology: is there a future for knockdown experiments in the era of genome editing? *J. Exp. Biol.,* **2019**, *222*(Pt 7), jeb175737. [http://dx.doi.org/10.1242/jeb.175737] [PMID: 30948498]

[81] El-Brolosy, M.A.; Stainier, D.Y.R. Genetic compensation: A phenomenon in search of mechanisms. *PLoS Genet.,* **2017**, *13*(7), e1006780. [http://dx.doi.org/10.1371/journal.pgen.1006780] [PMID: 28704371]

[82] Pena, I. A.; Roussel, Y.; Daniel, K.; Mongeon, K.; Johnstone, D.; Mendes, H. W.; Bosma, M.; Saxena, V.; Lepage, N.; Chakraborty, P. Pyridoxine-dependent epilepsy in zebrafish caused by Aldh7a1 deficiency. *Genetics*, **2017**, *207*(4), 1501-1518. [http://dx.doi.org/10.1534/genetics.117.300137]

[83] Xu, M.; Liu, D.; Dong, Z.; Wang, X.; Wang, X.; Liu, Y.; Baas, P.W.; Liu, M. Kinesin-12 influences axonal growth during zebrafish neural development. *Cytoskeleton (Hoboken),* **2014**, *71*(10), 555-563. [http://dx.doi.org/10.1002/cm.21193] [PMID: 25250533]

[84] Dong, Z.; Wu, S.; Zhu, C.; Wang, X.; Li, Y.; Chen, X.; Liu, D.; Qiang, L.; Baas, P.W.; Liu, M. CRISPR/Cas9-mediated Kif15 mutations accelerate axonal outgrowth during neuronal development and regeneration in zebrafish. *Traffic*, **2019**, *20*(1), 71-81. [http://dx.doi.org/10.1111/tra.12621] [PMID: 30411440]

[85] Gurung, S.; Asante, E.; Hummel, D.; Williams, A.; Feldman-Schultz, O.; Halloran, M.C.; Sittaramane, V.; Chandrasekhar, A. Distinct roles for the cell adhesion molecule contactin2 in the development and function of neural circuits in zebrafish. *Mech. Dev.,* **2018**, *152*, 1-12. [http://dx.doi.org/10.1016/j.mod.2018.05.005] [PMID: 29777776]

[86] Bose, P.; Armstrong, G. A. B.; Drapeau, P. Neuromuscular junctions abnormalities in a zebrafish loss-of-function model TDP-43. *J. Neurophysiol*., **2019**. *121*(1), 285-297.

[87] Volk, A. E.; Weishaupt, J. H.; Andersen, P. M.; Ludolph, A. C.; Kubisch, C. Current knowledge and recent insights into the genetic basis of amyotrophic lateral sclerosis. *Medizinische Genet.,* **2018**, *30*(2), 252–258. [http://dx.doi.org/10.1007/s11825-018-0185-3]

[88] Turner, A.N.; Andersen, R.S.; Bookout, I.E.; Brashear, L.N.; Davis, J.C.; Gahan, D.M.; Davis, J.C.; Gotham, J.P.; Hijaz, B.A.; Kaushik, A.S.; Mcgill, J.B.; Miller, V.L.; Moseley, Z.P.; Nowell, C.L.; Patel, R.K.; Rodgers, M.C.; Patel, R.K.; Shihab, Y.A.; Walker, A.P.; Glover, S.R.; Foster, S.D.; Challa, A.K. Analysis of novel domain-specific mutations in the zebrafish *ndr2*/*cyclops* gene generated using CRISPR-Cas9 RNPs. *J. Genet.,* **2018**, *97*(5), 1315-1325. [http://dx.doi.org/10.1007/s12041-018-1033-6] [PMID: 30555080]

[89] Jardin, N.; Giudicelli, F.; Ten Martín, D.; Vitrac, A.; De Gois, S.; Allison, R.; Houart, C.; Reid, E.; Hazan, J.; Fassier, C. BMP- and neuropilin 1-mediated motor axon navigation relies on spastin alternative translation. *Development,* **2018**, *145*(17), dev162701. [http://dx.doi.org/10.1242/dev.162701] [PMID: 30082270]

[90] Kozol, R.A.; Cukier, H.N.; Zou, B.; Mayo, V.; De Rubeis, S.; Cai, G.; Griswold, A.J.; Whitehead, P.L.; Haines, J.L.; Gilbert, J.R.; Cuccaro, M.L.; Martin, E.R.; Baker, J.D.; Buxbaum, J.D.; Pericak-Vance, M.A.; Dallman, J.E. Two knockdown models of the autism genes SYNGAP1 and SHANK3 in zebrafish produce similar behavioral phenotypes associated with embryonic disruptions of brain morphogenesis. *Hum. Mol. Genet.,* **2015**, *24*(14), 4006-4023. [http://dx.doi.org/10.1093/hmg/ddv138] [PMID: 25882707]

[91] Liu, C.X.; Li, C.Y.; Hu, C.C.; Wang, Y.; Lin, J.; Jiang, Y.H.; Li, Q.; Xu, X. CRISPR/Cas9-induced *shank3b* mutant zebrafish display autism-like behaviors. *Mol. Autism,* **2018**, *9*(1), 23. [http://dx.doi.org/10.1186/s13229-018-0204-x] [PMID: 29619162]

[92] Riché, R.; Drapeau, P.; Samarut, É. Glycine decarboxylase deficiency-induced motor dysfunction in zebrafish is rescued by counterbalancing glycine synaptic level. *JCI Insight*, **2018**. *3*(21), pii: 124642. [http://dx.doi.org/10.1172/jci.insight.124642]

[93] Khan, T.N.; Khan, K.; Sadeghpour, A.; Reynolds, H.; Perilla, Y.; McDonald, M.T.; Gallentine, W.B.; Baig, S.M.; Davis, E.E.; Katsanis, N. Mutations in NCAPG2 cause a severe neurodevelopmental syndrome that expands the phenotypic spectrum of sondensinopathies. *Am. J. Hum. Genet.,* **2019**, *104*(1), 94-111. [http://dx.doi.org/10.1016/j.ajhg.2018.11.017] [PMID: 30609410]

[94] Zelinka, C.P.; Sotolongo-Lopez, M.; Fadool, J.M. Targeted disruption of the endogenous zebrafish *rhodopsin* locus as models of rapid rod photoreceptor degeneration. *Mol. Vis.,* **2018**, *24*, 587-602. [PMID: 30210230]

[95] Grone, B.P.; Marchese, M.; Hamling, K.R.; Kumar, M.G.; Krasniak, C.S.; Sicca, F.; Santorelli, F.M.; Patel, M.; Baraban, S.C. Epilepsy, Behavioral Abnormalities, and Physiological Comorbidities in Syntaxin-Binding Protein 1 (STXBP1) Mutant Zebrafish. *PLoS One,* **2016**, *11*(3), e0151148. [http://dx.doi.org/10.1371/journal.pone.0151148] [PMID: 26963117]

[96] Zabinyakov, N.; Bullivant, G.; Cao, F.; Fernandez Ojeda, M.; Jia, Z.P.; Wen, X.Y.; Dowling, J.J.; Salomons, G.S.; Mercimek-Andrews, S. Characterization of the first knock-out aldh7a1 zebrafish model for pyridoxine-dependent epilepsy using CRISPR-Cas9 technology. *PLoS One,* **2017**, *12*(10), e0186645. [http://dx.doi.org/10.1371/journal.pone.0186645] [PMID: 29053735]

[97] Johnstone, D.L.; Al-Shekaili, H.H.; Tarailo-Graovac, M.; Wolf, N.I.; Ivy, A.S.; Demarest, S.; Roussel, Y.; Ciapaite, J.; van Roermund, C.W.T.; Kernohan, K.D.; Kosuta, C.; Ban, K.; Ito, Y.; McBride, S.; Al-Thihli, K.; Abdelrahim, R.A.; Koul, R.; Al Futaisi, A.; Haaxma, C.A.; Olson, H.; Sigurdardottir, L.Y.; Arnold, G.L.; Gerkes, E.H.; Boon, M.; Heiner-Fokkema, M.R.; Noble, S.; Bosma, M.; Jans, J.; Koolen, D.A.; Kamsteeg, E.J.; Drögemöller, B.; Ross, C.J.; Majewski, J.; Cho, M.T.; Begtrup, A.; Wasserman, W.W.; Bui, T.; Brimble, E.; Violante, S.; Houten, S.M.; Wevers, R.A.; van Faassen, M.; Kema, I.P.; Lepage, N.; Lines, M.A.; Dyment, D.A.; Wanders, R.J.A.; Verhoeven-Duif, N.; Ekker, M.; Boycott, K.M.; Friedman, J.M.; Pena, I.A.; van Karnebeek, C.D.M. PLPHP deficiency: clinical, genetic, biochemical, and mechanistic insights. *Brain,* **2019**, *142*(3), 542-559. [http://dx.doi.org/10.1093/brain/awy346] [PMID: 30668673]

[98] Weaver, C.J.; Terzi, A.; Roeder, H.; Gurol, T.; Deng, Q.; Leung, Y.F.; Suter, D.M. nox2/cybb deficiency affects Zebrafish retinotectal connectivity. *J. Neurosci.,* **2018**, *38*(26), 5854-5871. [http://dx.doi.org/10.1523/JNEUROSCI.1483-16.2018] [PMID: 29793976]

[99] Sedykh, I.; Keller, A.N.; Yoon, B.; Roberson, L.; Moskvin, O.V.; Grinblat, Y. Zebrafish Rfx4 controls dorsal and ventral midline formation in the neural tube. *Dev. Dyn.,* **2018**, *247*(4), 650-659. [http://dx.doi.org/10.1002/dvdy.24613] [PMID: 29243319]

[100] Yildiz, O.; Downes, G. B.; Sagerström, C. G. Zebrafish Prdm12b acts independently of Nkx6 . 1 repression to promote Eng1b Expression in the neural tube P1 domain, **2019**, 1–19.

[101] Hofmeister, W.; Pettersson, M.; Kurtoglu, D.; Armenio, M.; Eisfeldt, J.; Papadogiannakis, N.; Gustavsson, P.; Lindstrand, A. Targeted copy number screening highlights an intragenic deletion of WDR63 as the likely cause of human occipital encephalocele and abnormal CNS development in zebrafish. *Hum. Mutat.,* **2018**, *39*(4), 495-505. [http://dx.doi.org/10.1002/humu.23388] [PMID: 29285825]

[102] Perez, Y.; Bar-Yaacov, R.; Kadir, R.; Wormser, O.; Shelef, I.; Birk, O.S.; Flusser, H.; Birnbaum, R.Y. Mutations in the microtubule-associated protein MAP11 (C7orf43) cause microcephaly in humans and zebrafish. *Brain,* **2019**, *142*(3), 574-585. [http://dx.doi.org/10.1093/brain/awz004] [PMID: 30715179]

[103] Cai, S.; Chen, Y.; Shang, Y.; Cui, J.; Li, Z.; Li, Y. Knockout of zebrafish interleukin 7 receptor (IL7R) by the CRISPR/Cas9 system delays retinal neurodevelopment. *Cell Death Dis.,* **2018**, *9*(3), 273. [http://dx.doi.org/10.1038/s41419-018-0337-z] [PMID: 29449560]

[104] Puttonen, H.A.J.; Sundvik, M.; Semenova, S.; Shirai, Y.; Chen, Y.C.; Panula, P. Knockout of histamine receptor H3 alters adaptation to sudden darkness and monoamine levels in the zebrafish. *Acta Physiol. (Oxf.),* **2018**, *222*(3). [http://dx.doi.org/10.1111/apha.12981] [PMID: 29044927]

[105] Ashlin, T.G.; Blunsom, N.J.; Ghosh, M.; Cockcroft, S.; Rihel, J. Pitpnc1a regulates Zebrafish sleep and wake behavior through modulation of insulin-like growth factor signaling. *Cell Rep.,* **2018**, *24*(6), 1389-1396. [http://dx.doi.org/10.1016/j.celrep.2018.07.012] [PMID: 30089250]

[106] Küry, S.; Besnard, T.; Ebstein, F.; Khan, T.N.; Gambin, T.; Douglas, J.; Bacino, C.A.; Craigen, W.J.; Sanders, S.J.; Lehmann, A.; Latypova, X.; Khan, K.; Pacault, M.; Sacharow, S.; Glaser, K.; Bieth, E.; Perrin-Sabourin, L.; Jacquemont, M.L.; Cho, M.T.; Roeder, E.; Denommé-Pichon, A.S.; Monaghan, K.G.; Yuan, B.; Xia, F.; Simon, S.; Bonneau, D.; Parent, P.; Gilbert-Dussardier, B.; Odent, S.; Toutain, A.; Pasquier, L.; Barbouth, D.; Shaw, C.A.; Patel, A.; Smith, J.L.; Bi, W.; Schmitt, S.; Deb, W.; Nizon, M.; Mercier, S.; Vincent, M.; Rooryck, C.; Malan, V.; Briceño, I.; Gómez, A.; Nugent, K.M.; Gibson, J.B.; Cogné, B.; Lupski, J.R.; Stessman, H.A.F.; Eichler, E.E.; Retterer, K.; Yang, Y.; Redon, R.; Katsanis, N.; Rosenfeld, J.A.; Kloetzel, P.M.; Golzio, C.; Bézieau, S.; Stankiewicz, P.; Isidor, B. *De Novo* disruption of the proteasome regulatory subunit PSMD12 causes a syndromic neurodevelopmental Disorder. *Am. J. Hum. Genet.,* **2017**, *100*(2), 352-363. [http://dx.doi.org/10.1016/j.ajhg.2017.01.003] [PMID: 28132691]

[107] Du, W.J.; Zhang, R.W.; Li, J.; Zhang, B.B.; Peng, X.L.; Cao, S.; Yuan, J.; Yuan, C.D.; Yu, T.; Du, J.L. The locus coeruleus modulates intravenous general anesthesia of Zebrafish *via* a cooperative mechanism. *Cell Rep.,* **2018**, *24*(12), 3146-3155.e3. [http://dx.doi.org/10.1016/j.celrep.2018.08.046] [PMID: 30231998]

[108] Zhang, H.; Zhang, Q.; Gao, G.; Wang, X.; Wang, T.; Kong, Z.; Wang, G.; Zhang, C.; Wang, Y.; Peng, G. UBTOR/KIAA1024 regulates neurite outgrowth and neoplasia through mTOR signaling. *PLoS Genet.,* **2018**, *14*(8), e1007583. [http://dx.doi.org/10.1371/journal.pgen.1007583] [PMID: 30080879]

[109] Bar Yaacov, R.; Eshel, R.; Farhi, E.; Shemuluvich, F.; Kaplan, T.; Birnbaum, R.Y. Functional Characterization of the ZEB2 Regulatory Landscape. *Hum. Mol. Genet*., **2019**, *28*(9),1487-1497.

[110] Lazcano, I.; Rodríguez-Ortiz, R.; Villalobos, P.; Martínez-Torres, A.; Solís-Saínz, J.C.; Orozco, A. Knock-down of specific thyroid hormone receptor Isoforms impairs body plan development in Zebrafish. *Front. Endocrinol., (Lausanne),* **2019**, *10*, 156. [http://dx.doi.org/10.3389/fendo.2019.00156] [PMID: 30930855]

[111] Puttonen, H.A.J.; Sundvik, M.; Semenova, S.; Shirai, Y.; Chen, Kuil, L.E.; Oosterhof, N.; Geurts, S.N.; van der Linde, H.C.; Meijering, E.; van Ham, T.J. Reverse genetic screen reveals that Il34 facilitates yolk sac macrophage distribution and seeding of the brain. *Dis. Model. Mech*., **2019**, *12*(3), pii: dmm037762. [http://dx.doi.org/10.1242/dmm.037762] [PMID: 30765415]

[112] Jobst-Schwan, T.; Schmidt, J.M.; Schneider, R.; Hoogstraten, C.A.; Ullmann, J.F.P.; Schapiro, D.; Majmundar, A.J.; Kolb, A.; Eddy, K.; Shril, S.; Braun, D.A.; Poduri, A.; Hildebrandt, F. Acute multi-sgRNA knockdown of KEOPS complex genes reproduces the microcephaly phenotype of the stable knockout zebrafish model. *PLoS One,* **2018**, *13*(1), e0191503. [http://dx.doi.org/10.1371/journal.pone.0191503] [PMID: 29346415]

[113] Braun, D.A.; Rao, J.; Mollet, G.; Schapiro, D.; Daugeron, M-C.; Tan, W.; Gribouval, O.; Boyer, O.; Revy, P.; Jobst-Schwan, T.; Schmidt, J.M.; Lawson, J.A.; Schanze, D.; Ashraf, S.; Ullmann, J.F.P.; Hoogstraten, C.A.; Boddaert, N.; Collinet, B.; Martin, G.; Liger, D.; Lovric, S.; Furlano, M.; Guerrera, I.C.; Sanchez-Ferras, O.; Hu, J.F.; Boschat, A.C.; Sanquer, S.; Menten, B.; Vergult, S.; De Rocker, N.; Airik, M.; Hermle, T.; Shril, S.; Widmeier, E.; Gee, H.Y.; Choi, W.I.; Sadowski, C.E.; Pabst, W.L.; Warejko, J.K.; Daga, A.; Basta, T.; Matejas, V.; Scharmann, K.; Kienast, S.D.; Behnam, B.; Beeson, B.; Begtrup, A.; Bruce, M.; Ch'ng, G.S.; Lin, S.P.; Chang, J.H.; Chen, C.H.; Cho, M.T.; Gaffney, P.M.; Gipson, P.E.; Hsu, C.H.; Kari, J.A.; Ke, Y.Y.; Kiraly-Borri, C.; Lai, W.M.; Lemyre, E.; Littlejohn, R.O.; Masri, A.; Moghtaderi, M.; Nakamura, K.; Ozaltin, F.; Praet, M.; Prasad, C.; Prytula, A.; Roeder, E.R.; Rump, P.; Schnur, R.E.; Shiihara, T.; Sinha, M.D.; Soliman, N.A.; Soulami, K.; Sweetser, D.A.; Tsai, W.H.; Tsai, J.D.; Topaloglu, R.; Vester, U.; Viskochil, D.H.; Vatanavicharn, N.; Waxler, J.L.; Wierenga, K.J.; Wolf, M.T.F.; Wong, S.N.; Leidel, S.A.; Truglio, G.; Dedon, P.C.; Poduri, A.; Mane, S.; Lifton, R.P.; Bouchard, M.; Kannu, P.; Chitayat, D.; Magen, D.; Callewaert, B.; van Tilbeurgh, H.; Zenker, M.; Antignac, C.; Hildebrandt, F. Mutations in KEOPS-complex genes cause nephrotic syndrome with primary microcephaly. *Nat. Genet.,* **2017**, *49*(10), 1529-1538. [http://dx.doi.org/10.1038/ng.3933] [PMID: 28805828]

[114] Kimura, Y.; Hisano, Y.; Kawahara, A.; Higashijima, S. Efficient generation of knock-in transgenic zebrafish carrying reporter/driver genes by CRISPR/Cas9-mediated genome engineering. *Sci. Rep.,* **2014**, *4*, 6545. [http://dx.doi.org/10.1038/srep06545] [PMID: 25293390]

[115] Ota, S.; Taimatsu, K.; Yanagi, K.; Namiki, T.; Ohga, R.; Higashijima, S.I.; Kawahara, A.; Jinek, M.; Cong, L.; Mali, P. Functional visualization and disruption of targeted genes using CRISPR/Cas9-mediated eGFP reporter integration in zebrafish. *Sci. Rep.,* **2016**, *6*, 34991. [http://dx.doi.org/10.1038/srep34991] [PMID: 27725766]

[116] Wu, R.S.; Lam, I.I.; Clay, H.; Duong, D.N.; Deo, R.C.; Coughlin, S.R. A rapid method for directed gene knockout for screening in G0 Zebrafish. *Dev. Cell,* **2018**, *46*(1), 112-125.e4. [http://dx.doi.org/10.1016/j.devcel.2018.06.003] [PMID: 29974860]

[117] Chen, T.W.; Wardill, T.J.; Sun, Y.; Pulver, S.R.; Renninger, S.L.; Baohan, A.; Schreiter, E.R.; Kerr, R.A.; Orger, M.B.; Jayaraman, V.; Looger, L.L.; Svoboda, K.; Kim, D.S. Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature,* **2013**, *499*(7458), 295-300. [http://dx.doi.org/10.1038/nature12354] [PMID: 23868258]

[118] Ahrens, M.B.; Orger, M.B.; Robson, D.N.; Li, J.M.; Keller, P.J. Whole-brain functional imaging at cellular resolution using light-sheet microscopy. *Nat. Methods,* **2013**, *10*(5), 413-420. [http://dx.doi.org/10.1038/nmeth.2434] [PMID: 23524393]

[119] Farrar, M.J.; Kolkman, K.E.; Fetcho, J.R. Features of the structure, development, and activity of the zebrafish noradrenergic system explored in new CRISPR transgenic lines. *J. Comp. Neurol.,* **2018**, *526*(15), 2493-2508. [http://dx.doi.org/10.1002/cne.24508] [PMID: 30070695]

[120] Motta-Mena, L.B.; Reade, A.; Mallory, M.J.; Glantz, S.; Weiner, O.D.; Lynch, K.W.; Gardner, K.H. An optogenetic gene expression system with rapid activation and deactivation kinetics. *Nat. Chem. Biol.,* **2014**, *10*(3), 196-202. [http://dx.doi.org/10.1038/nchembio.1430] [PMID: 24413462]

[121] Buckley, C.E.; Moore, R.E.; Reade, A.; Goldberg, A.R.; Weiner, O.D.; Clarke, J.D.W. Reversible optogenetic control of subcellular protein localization in a live vertebrate embryo. *Dev. Cell,* **2016**, *36*(1), 117-126. [http://dx.doi.org/10.1016/j.devcel.2015.12.011] [PMID: 26766447]

[122] Reade, A.; Motta-Mena, L.B.; Gardner, K.H.; Stainier, D.Y.; Weiner, O.D.; Woo, S. TAEL: a zebrafish-optimized optogenetic gene expression system with fine spatial and temporal control. *Development,* **2017**, *144*(2), 345-355. [http://dx.doi.org/10.1242/dev.139238] [PMID: 27993986]

[123] Putri, R.R.; Chen, L. Spatiotemporal control of zebrafish (Danio rerio) gene expression using a light-activated CRISPR activation system. *Gene,* **2018**, *677*(July), 273-279. [http://dx.doi.org/10.1016/j.gene.2018.07.077] [PMID: 30077009]

[124] Nihongaki, Y.; Yamamoto, S.; Kawano, F.; Suzuki, H.; Sato, M. CRISPR-Cas9-based photoactivatable transcription system. *Chem. Biol.,* **2015**, *22*(2), 169-174. [http://dx.doi.org/10.1016/j.chembiol.2014.12.011] [PMID: 25619936]

[125] Yang, H.H.; St-Pierre, F. Genetically Encoded Voltage Indicators: Opportunities and Challenges. *J. Neurosci.,* **2016**, *36*(39), 9977-9989. [http://dx.doi.org/10.1523/JNEUROSCI.1095-16.2016] [PMID: 27683896]

[126] Kibat, C.; Krishnan, S.; Ramaswamy, M.; Baker, B.J.; Jesuthasan, S. Imaging voltage in zebrafish as a route to characterizing a vertebrate functional connectome: promises and pitfalls of genetically encoded indicators. *J. Neurogenet.,* **2016**, *30*(2), 80-88. [http://dx.doi.org/10.1080/01677063.2016.1180384] [PMID: 27328843]

[127] Benedetti, L.; Ghilardi, A.; Prosperi, L.; Francolini, M.; Del Giacco, L. Biosensing motor neuron membrane potential in live zebrafish embryos. *J. Vis. Exp*., **2017**, *124*, pp. 1-8.

[128] Miyazawa, H.; Okumura, K.; Hiyoshi, K.; Maruyama, K.; Kakinuma, H.; Amo, R.; Okamoto, H.; Yamasu, K.; Tsuda, S. Optical interrogation of neuronal circuitry in zebrafish using genetically encoded voltage indicators. *Sci. Rep.,* **2018**, *8*(1), 6048. [http://dx.doi.org/10.1038/s41598-018-23906-1] [PMID: 29662090]

[129] Breacker, C.; Barber, I.; Norton, W.H.J.; McDearmid, J.R.; Tilley, C.A. A Low-Cost Method of Skin Swabbing for the Collection of DNA Samples from Small Laboratory Fish. *Zebrafish,* **2017**, *14*(1), 35-41. [http://dx.doi.org/10.1089/zeb.2016.1348] [PMID: 27788059]

[130] Borck, G.; Hög, F.; Dentici, M.L.; Tan, P.L.; Sowada, N.; Medeira, A.; Gueneau, L.; Thiele, H.; Kousi, M.; Lepri, F.; Wenzeck, L.; Blumenthal, I.; Radicioni, A.; Schwarzenberg, T.L.; Mandriani, B.; Fischetto, R.; Morris-Rosendahl, D.J.; Altmüller, J.; Reymond, A.; Nürnberg, P.; Merla, G.; Dallapiccola, B.; Katsanis, N.; Cramer, P.; Kubisch, C. BRF1 mutations alter RNA polymerase III-dependent transcription and cause neurodevelopmental anomalies. *Genome Res.,* **2015**, *25*(2), 155-166. [http://dx.doi.org/10.1101/gr.176925.114] [PMID: 25561519]

[131] Hofmeister, W.; Pettersson, M.; Kurtoglu, D.; Armenio, M.; Eisfeldt, J.; Papadogiannakis, N.; Gustavsson, P.; Lindstrand, A. Targeted copy number screening highlights an intragenic deletion of WDR63 as the likely cause of human occipital encephalocele and abnormal CNS development in zebrafish. *Hum. Mutat.,* **2018**, *39*(4), 495-505. [http://dx.doi.org/10.1002/humu.23388] [PMID: 29285825]