Université de Montréal

"Functional characterization of INTS11 loss-of-function in zebrafish"

Par

Aveeva Herold

Département de Neurosciences, Faculté de Médecine

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"Functional characterization of INTS11 loss-of-function in zebrafish"

Présenté par

Aveeva Herold

A été évaluée par un jury composé des personnes suivantes

Dr. Adriana Di Polo

Président-rapporteur

Dr. Éric Samarut

Directeur de recherche

Dr. Gary Amstrong

Membre du jury

Résumé

Le gène INTS11 est une sous-unité catalytique du complexe Integrator qui joue un rôle central dans le traitement de divers ARN naissants. Récemment, des patients présentant des mutations de perte de fonction dans le gène INTS11 ont été signalés comme ayant des problèmes neurodéveloppementaux graves, des problèmes ataxiques et des retards de développement globaux. À ce jour, aucune mutation dans INTS11 n'a été liée à des maladies humaines, et aucune preuve ne soutient leur rôle dans des problèmes neurodéveloppementaux. Par conséquent, nous avons développé un modèle INTS11 knock-out (KO) F0 CRISPRant chez le poisson-zèbre pour caractériser fonctionnellement les mutations de perte de fonction de ce gène in vivo. Nos larves INTS11-KO présentent une accumulation accrue de snARN mal traités, confirmant la perturbation de la fonction du gène. De plus, les larves INTS11-KO meurent prématurément à 14 jours et présentent un phénotype comportemental aberrant, similaire à d'autres modèles génétiques de poisson-zèbre des troubles neurodéveloppementaux. Aussi, les larves INTS11-KO présentent une réduction de la taille du cerveau avec une réduction du contenu neuronal. Enfin, nos résultats d'immunomarquage ont révélé une réduction de la taille du cervelet chez les larves INTS11-KO. Dans l'ensemble, ces données soutiennent le rôle d'INTS11 dans le développement cérébral et sont cohérentes avec les retards neurodéveloppementaux décrits chez les patients présentant des mutations délétères dans ce gène. Notre étude montre comment des organismes modèles simples tels que le poisson-zèbre peuvent aider à caractériser l'étiologie génétique des troubles génétiques. Les résultats de nos recherches pourraient contribuer à des diagnostics plus précis et

ouvrir la voie à la découverte de mécanismes pathogènes clés qui pourraient être exploités pour le développement de traitements pour les patients présentant des mutations dans INTS11.

Mots-Clés: INTS11, Caractérisation Fonctionnelle, Poisson-Zèbre, Médecine De Précision, Neurodéveloppement.

Abstract

The *INTS11* gene is a catalytic subunit of the Integrator complex that plays a central role in processing various nascent RNAs. Recently, patients with loss-of-function mutations in the *INTS11* gene have been reported to have severe neurodevelopmental issues, ataxic problems, and global developmental delays. To date, mutations in *INTS11* have not been linked to human diseases, and no evidence supports their role in neurodevelopmental problems. Therefore, we developed an *ints11* F0 CRISPRant knock-out (KO) model in zebrafish to functionally characterize loss-of-function mutations in this gene *in vivo*. Our *ints11*-KO larvae exhibited an increased accumulation of unprocessed snRNAs, confirming the disruption of the *ints11* function. Moreover, *ints11*-KO larvae die prematurely by 14 days of age and display an aberrant behavioural phenotype, similar to other zebrafish genetic models of neurodevelopmental disorders. Furthermore, *ints11*-KO larvae show reduced brain size with reduced neuronal content. Finally, immunostaining results revealed a reduction in cerebellum size in our *ints11*-KO. Altogether, these data support the role of *INTS11* in brain development and are consistent with the neurodevelopment delays described in patients with deleterious mutations in this gene. Our study shows how simple model organisms like zebrafish can help characterize the genetic etiology of genetic disorders. The results from our research could aid in more accurate diagnoses and open the path to unveiling key pathogenic mechanisms that could be leveraged for the development of treatment for patients with mutations in *INTS11*.

Keywords: *INTS11*, functional characterization, zebrafish, precision medicine, neurodevelopment.

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List of Abbreviations

AED: anti-epileptic drugs

Cas-9: CRISPR-associated protein 9

CNS: central nervous system

CRISPR: clustered regulatory-interspaced short palindromic

Dfp: days post fertilization

GFP: Green Fluorescent Protein

GoF: Gain of Function

gRNAs: guide RNAs

Hpf: hours post-fertilization

HRM: High-Resolution Melting

INT: Integrator complex

INTS11: Integrator complex subunit 11

KO - knockout

LoF: Loss of Function

loxP: Locus of X-over P1

MO: Morpholino antisense oligomers

mRNA: messenger RNA

NHEJ: Non-Homologous End Joining

PAV7: parvalbumin-7

RFP: Red Fluorescent Protein

RNA: ribonucleic acid

RNAPII: RNA Polymerase II

SNPs: Single Nucleotide Polymorphisms

snRNPs: small nuclear ribonucleoproteins

SOSS Complex: Sensor of Single-Stranded DNA Complex

U snRNAs: uridine rich small nuclear RNAs

VUS: Variants of Unknown Significance

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CHAPTER 1: INTRODUCTION

1. Introduction to precision medicine

Precision medicine is a prominent subject within applied sciences, modern biomedicine, and biomedical studies. However, the term 'precision medicine' encompasses a wide range of definitions that can potentially make its understanding complex, lack clarity, and cause some degree of confusion in comprehension. As a widely accepted definition, precision medicine, also called personalized medicine, is a form of medicine that considers the individual variations in genes, environments, and lifestyle of an individual to prevent, diagnose or treat disease (Delpierre and Lefèvre, 2023)

1.1 Historical overview of precision medicine

The term “precision medicine” may be new, leading some people to believe it is an invention in the 21st century. However, precision medicine dates back to ancient times, when physicians always tailored their medical recommendations to individual factors such as age, gender, and other patients’ lifestyle specificities. The first reports on adapting medicine to an individual’s health status can be traced back to ancient Egypt's history. During this period, the doctors categorized the diseases based on different body parts, aiming to better understand the illnesses and ultimately achieve improved therapeutic outcomes (Visvikis-Siest et al., 2020). As modern medicine developed in the 20th century, precision medicine began to rely on accurate molecular cues such as rhesus factors that define blood types. For instance, an individual requiring a blood transfusion is not administered blood from a randomly selected donor; instead, the donor’s blood type is precisely matched to the recipient to reduce the risk of complications. Thus, it quickly

became practical to group patients based on their blood type to improve the success of blood transfusions (Klein et al., 2015).

Fast forward to the 21st century, the personalized aspect of medicine has become more evident following human genome sequencing. It is now possible to correlate the genetic profile of individuals to their overall health and treatment responsiveness (Brittain et al., 2017; Carrasco-Ramiro et al., 2017). This is the basis of the modern definition of precision medicine, *"which aims to enhance diagnosis and prognosis while tailoring the medication to each individual based on genetic variations"*(McAlister et al., 2017).

As it is not easy to derive a simple definition of precision medicine, I recently co-wrote a review in which we have described the main aspects of what we can refer to as precision medicine, which will be summarized in the next section (Ochenkowska et al., 2022). Of note is that the full review manuscript is attached in Appendix B.

1.2 Various Aspects of Precision Medicine

In our review, we categorized precision medicine into four main areas: (1) tailoring medication, (2) advancing drug discovery, (3) identifying biomarkers, and (4) improving diagnostic practices (Figure 1).

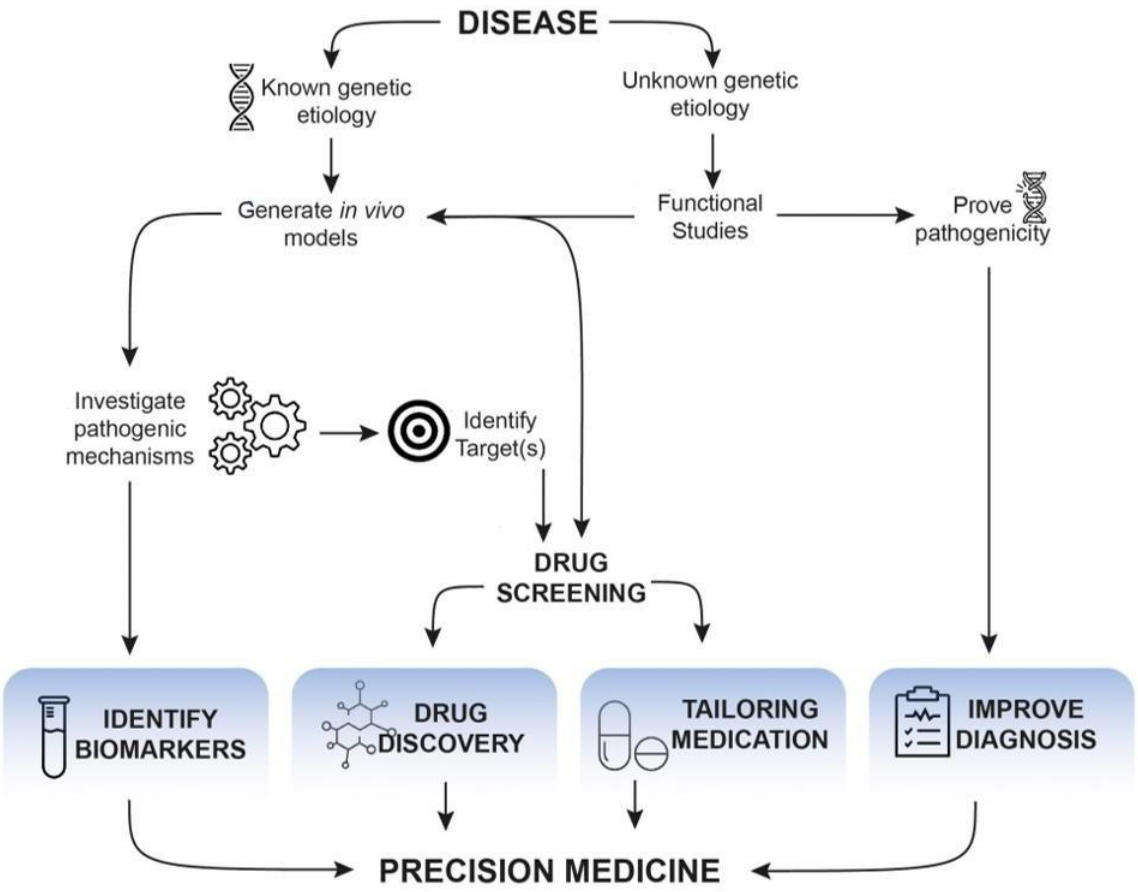

Figure 1: Flowchart illustrating key aspects of precision medicine

(taken from Ochenkowska et al., 2022*)*

1.2.1 Tailoring medication

Tailoring medication involves optimizing treatments based on genetic variations (pharmacogenomics) or treating individuals separately based on their genetic etiology.

1.2.1.1 Pharmacogenomics

Pharmacogenomics is a critical concept in tailoring medication that studies how an individual's genetic makeup influences their response to medications. It combines insights from pharmacology (the study of how drugs work) and genomics (the analysis of genes and their functions) to optimize drug treatments based on individual genetic variations.

Genetic variations among individuals can significantly impact drug metabolism, efficacy, and adverse reactions (Ahmed et al., 2016). For example, a patient carrying a variant allele of one of these genes could potentially face severe and, in some cases, life-threatening adverse events upon exposure to specific medications. On the other hand, the patient's particular metabolic genetic profile could lead to a quick degradation of the active principle, thus minimizing the treatment benefit. As a matter of fact, utilizing pharmacogenomics as a clinical tool can enhance treatment outcomes by reducing adverse reactions and optimizing drug selection across a spectrum of medical conditions (Hockings et al., 2020).

1.2.1.2 Future of tailoring medication

Moreover, our dream of precision medicine could also be to provide personalized treatment to individuals based on their genetic variation. To understand this concept of precision medicine, let's examine the genetic component of the neurological condition epilepsy. Epilepsy encompasses a spectrum of disorders and displays heterogeneity in clinical presentation and etiology, complicating seizure prevention and treatment. Although 28 anti-epileptic drugs (AED) are available in the market, treatment response is often unpredictable, and approximately one-third of patients fail to gain complete seizure control with pharmacotherapy alone (Chen et al., 2018). Furthermore, selecting the most suitable anti-epileptic drugs depends on medical professionals' empirical trial and error method. An inappropriate first medication can severely affect the efficacy of further treatments (Pawluski et al., 2018). This underlines the need to identify which AED works best for each patient as quickly as possible.

Further, the genotype-phenotype correlations are very complex since different mutations, even at the level of the same gene, can lead to varying types of seizures (Johannesen et al., 2016; Shen et al., 2017; Gontika et al., 2017). As a result, each epileptic patient might have to be considered unique in treatment responsiveness. As the traditional one-size-fits-all approach does not apply to the medical treatment of epilepsy, there is a need for patient-personalized genetic avatars that could tailor the treatment at the level of individual patients/mutations (Ochenkowska et al., 2022).

1.2.2 Drug discovery

Precision medicine can also be an attempt to understand the underlying genetic cause of the disease in individual patients. If the genetic etiology of a patient's disease is known, further *in vivo* models can be generated for better deciphering the disease pathogenesis. This will eventually lead to identifying potential drug targets and developing new drugs (drug discovery) (Dugger et al., 2018). In addition, these *in vivo* genetic models can also help identify potential novel disease biomarkers that can be further validated in patient populations (Figure 1).

Drug discovery is generally a lengthy process, from the initial discovery of an active ingredient (drug-like compound) to the development process, including testing in animal models and humans (Zon and Peterson, 2005).

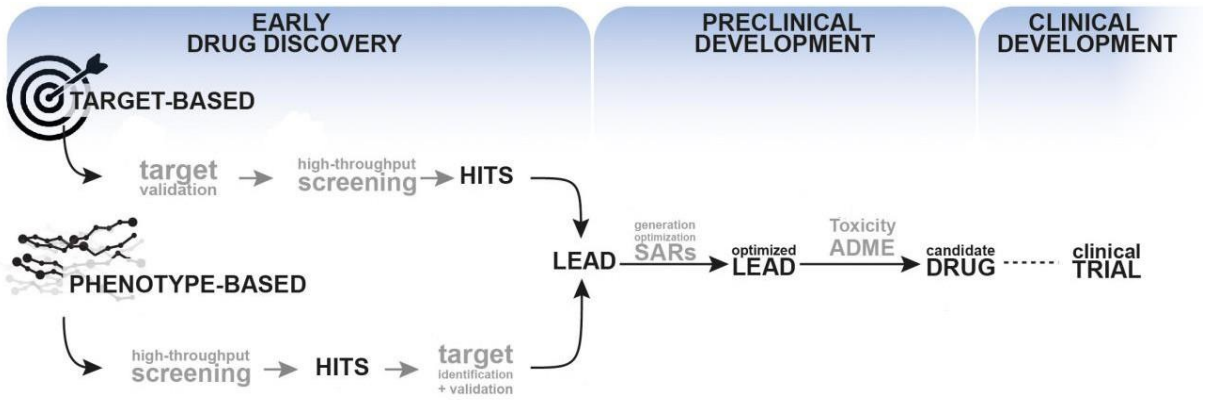

Figure 2- Various stages of drug development, from early discovery to preclinical development *(taken from* Ochenkowska et al., 2022*).*

There are various stages in the drug discovery process, such as early drug discovery, preclinical development, and clinical development. As described in Figure 2, the end goal of the early drug discovery phase is to identify an active molecule (*lead*) that has the potential to develop as a drug. Early drug discovery can either be a target-based approach, where the molecular target hypothesized to have an essential role in disease is identified and validated or a phenotype-based approach, where compounds modifying a disease phenotype are identified without prior knowledge of a specific target. Once the lead compound is identified, it undergoes preclinical studies involving the optimization of lead compounds, as well as assessment of ADME (absorption, distribution, metabolism, and excretion) and toxicity in various preclinical models such as mice, rats, zebrafish, cells cultures and non-human primates. The candidate drug that successfully passes the preclinical studies is subjected to human clinical trials (Hughes et al., 2011; Mohs and Greig, 2017).

1.2.3 Improving diagnosis

Precision medicine also aims to tailor treatment and improve diagnosis based on an individual's genetic makeup. However, when the genetic cause of a disease is unknown,

diagnosing and treating it becomes more challenging. This represents one of the primary challenges that precision medicine faces - improving and accelerating disease diagnosis. Moreover, next-generation sequencing has enabled the identification of specific loci, genes, and associated mutations linked to various diseases (Phan et al., 2006). The number of new disease-causing genetic variants has increased, and these new genomic data are crucial for understanding the etiology of human diseases (Koboldt et al., 2013). However, as the quantity and complexity of genetic information increase, the number of Variants of Unknown Significance (VUS) rises as well (Koboldt et al., 2013; Federici and Soddu, 2020). These genetic variations, for which we lack knowledge about molecular and physiological consequences, pose a significant challenge in genetic diagnosis and counselling (Hoffman-Andrews, 2017). In fact, according to the American College of Medical Genetics and Genomics (ACMG) standards, VUS should not be utilized in clinical decision-making. Unfortunately, VUS are often prevalent in sequencing-based clinical genetic tests (Richards et al., 2015). In enhancing diagnosis through precision medicine, addressing the challenge of VUS is critical (Hoffman-Andrews, 2017; Joynt et al., 2022). This involves advancing techniques and approaches in functional genomics and characterization. By better elucidating the functional effects of VUS and their role in disease pathways, we can reduce the uncertainty surrounding these variants and improve their clinical interpretation. The upcoming section will explore utilizing functional studies to characterize and validate pathogenicity for these VUS. This ultimately aids in more accurate and tailored diagnoses, advancing personalized patient care goals.

1.3 Functional characterization for pathogenicity validation

Functional characterization refers to understanding how a specific gene or genetic variant contributes to the normal functioning of an organism or how it might lead to a particular disease or disorder. It is a crucial step in understanding the significance of genetic variations, especially in those with uncertain clinical implications and cases with an unknown genetic etiology. This characterization helps researchers determine whether a genetic variant is associated with a particular disease. It mainly investigates the functional effects of these variants to determine their roles in disease development. It helps bridge the gap between genetic information and the actual biological consequences, aiding in accurate diagnosis, prognosis, and the development of targeted therapies. Various approaches for functional characterization are performed in research laboratories, including experimental methods such as *in vitro* assays and *in vivo* studies, as well as computational methods such as *in silico* tools.

At the scale of variant identification, computational prediction tools like SIFT (Ng and Henikoff, 2003), CADD (Kircher et al., 2014), SNAP2 (Hecht et al., 2015), and Polyphen (Adzhubei et al., 2010) can help predict and anticipate variant pathogenicity (Thusberg and Vihinen, 2009). However, recent studies have shown a clear discrepancy between missense variants' predicted and experimentally-validated effects. The results from several computational tools frequently conflict because of differences in the prediction algorithms (Sadowski et al., 2017) (Miosge et al., 2015). Additionally, most tools have shown poor accuracy and a bias toward false positives for pathogenicity, frequently leading to an over-prediction of pathogenicity (Sun et al., 2016). This

emphasizes the need for a wide variety of *in vitro* and *in vivo* model assays for functional characterization.

In-vitro studies mainly involve experiments conducted outside a living organism. Cell cultures and biochemical assays are commonly used to assess the functional consequences of genetic variants. For example, researchers have used a combination of in vitro assays in the *PALB2* gene, which is associated with varying levels of risk for breast, ovarian and pancreatic cancers. The impact of specific *PALB2* VUS has been accessed at the level of individual patients on the function of the protein in DNA repair, cell cycle regulation and the control of cellular levels of reactive oxygen species. This functional characterization of VUS can be useful for estimating cancer risk and determining how each patient will respond to cancer treatments. (Millot et al., 2012). Another study focuses on Wilson's disease, caused by a mutation in the *ATP7B* gene. The researchers performed *in vitro* assays, investigating fundamental aspects like protein levels, copper export capacity, and the cellular localization of *ATP7B* VUS. The results from an *in vitro* study combined with a structural analysis on an *ATP7B* protein model helped to reclassify certain *ATP7B* VUS as likely pathogenic or pathogenic (Stalke et al., 2023). These studies exemplify how *in vitro* functional characterization can aid in understanding genetic variants' impact on diseases. However, these *in vitro* studies may not fully replicate the complex physiological environment of living organisms, which requires additional validation *in vivo* models.

1.3.1 Use of model organisms in variant functionalization

Using simple animal models like worms, fruit flies, and fish is crucial to overcome the limitations of *in vitro* and *sillico* studies for the functional characterization of variants. These models help to bridge the gap between genetic diagnosis and functional studies. It

also enables studying the effects of genetic variants in a complex biological context. For example, a study by Lange et al. used *Caenorhabditis elegans* as an *in vivo* model for identifying missense VUS associated with *TMEM67*, a gene associated with ciliopathies. They have employed CRISPR/Cas9 gene editing to introduce patient variants of the *TMEM67* gene into the nematode's orthologous gene (mks-3) and conducted quantitative assays to evaluate sensory cilia structure and function. With the results, they were able to classify the 8 missense VUS as benign (3) and pathogenic (5) (Lange et al., 2022). In another study, using *Drosophila melanogaster* (fruit fly) as a model, the authors assessed approximately 100 human *PTEN* (tumour suppressor gene) variants for their impact on cellular growth and proliferation. Results from the study were aligned with known pathogenic and benign variants (Ganguly et al., 2021). These studies highlight the relevance and advantages of using simple animal models for rapid functional studies.

In recent years, zebrafish (*Danio rerio*) have grown in importance as a tool for functional studies. The latest advancements in bioengineering techniques enable researchers to study gene functions and the impacts of mutations directly in vivo in zebrafish larvae. The following section will explore the advantages of using zebrafish for functional characterization.

2. Zebrafish as a model

The zebrafish is a tropical freshwater fish native to Southeast Asia, which has emerged as a dynamic model organism for biological research. It has been used for decades as a classical developmental biology model (Streisinger et al., 1981). Due to the experimental advantages of zebrafish, they are a widely used model in various research

areas, such as neurology, genetics, nutrition, physiology toxicology, disease modelling, developmental biology, and drug discovery (Teame et al., 2019).

Firstly, zebrafish are small (about 3 cm) and require simple husbandry skills, making them easy to maintain at a low cost. They also undergo rapid external development with a high fecundity rate, with adult female zebrafish yielding 100-200 fertilized eggs. Secondly, the optical transparency of the embryos allows us to visualize the internal organ development. The embryos are also amenable to genetic manipulation, enabling researchers to precisely modify the genes (Cornet et al., 2018). Finally, after the zebrafish genome sequencing project was finished in 2013, it was found that there were significant similarities between the zebrafish and human genomes, with about 70% of human genes having orthologs in zebrafish (Rubbini et al., 2020).

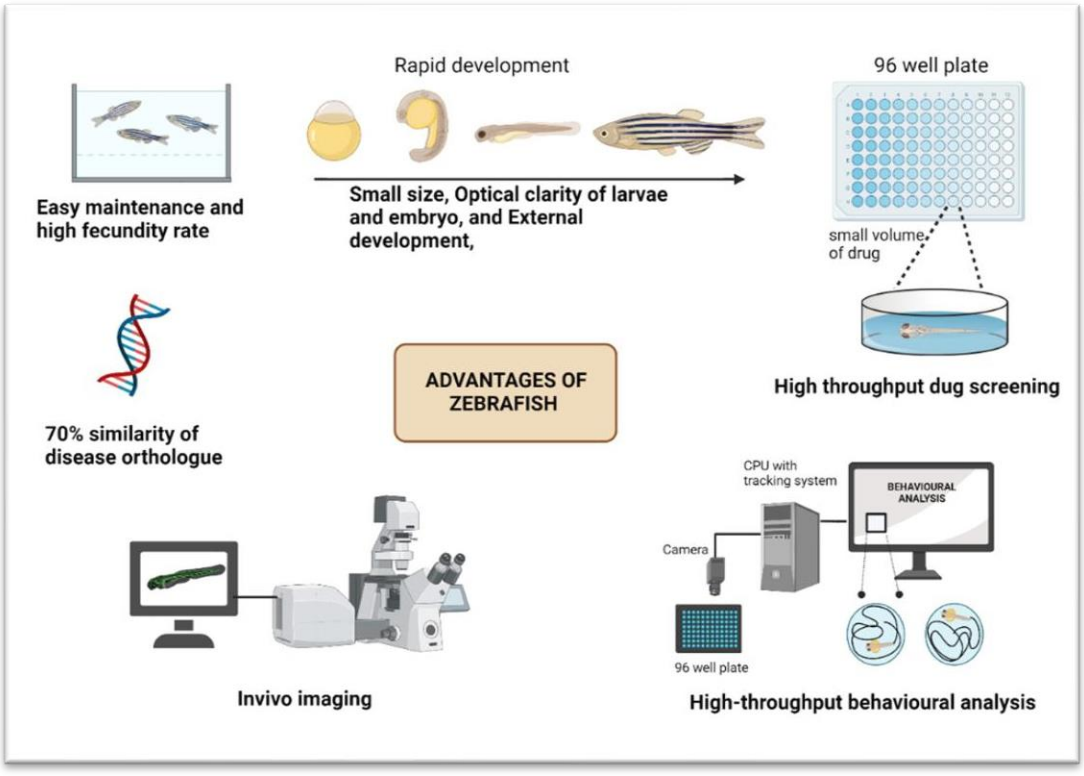

Figure 3: Main advantages of zebrafish in biological research

While these advantages (as illustrated in Figure 3) are similar to those found in other standard invertebrate models, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, zebrafish are complemented by relevant structural similarities to mammalian physiology. As zebrafish possess an integrated nervous system, their brain has structures that are homologous to those found in mammals. They also have cellular and synaptic networks and functions equivalent to mammals. This makes zebrafish a well-known model for investigating biological issues, particularly central nervous system (CNS) development (Panula et al., 2010; Corradi and Filosa, 2021). Moreover, zebrafish larvae exhibit a diverse range of complex behaviours within just a few days after fertilization, enabling them as a tool to perform high throughput behaviour analysis. Moreover, their small size and easy handling make them a great model for preclinical drug discovery. Ultimately, this makes zebrafish a powerful tool for understanding developmental processes and disease mechanisms, behavioural studies, toxicity, and drug screening (MacRae and Peterson, 2015).

2.1 Zebrafish as a Functional Characterization Toolbox

Zebrafish serve as a valuable model for characterizing gene function and ultimately validating the pathogenicity of specific genetic variants. As discussed earlier, various advantages make zebrafish a powerful model. The well-characterized genome is one key feature that makes zebrafish exclusively useful for functional characterization. The Zebrafish genome shares a high degree of similarity with the human genome, with approximately 83% of the human disease-causing genes having an ortholog in zebrafish, making it particularly relevant for studying human diseases (Howe et al., 2013). This makes them an excellent tool for modelling a wide array of diseases. Many well-established

models already exist for cardiovascular diseases (Tessadori et al., 2018), skeletal diseases (Wu et al., 2019), cancer (Mayrhofer and Mione, 2016), and particularly neurodevelopmental disorders (Sakai et al., 2018).

Furthermore, zebrafish reproduce externally, and their embryos are easy to manipulate by injecting genetic material during the earliest stage of development. This enables zebrafish as an ideal platform to carry out a variety of functional characterization procedures, depending on the class of the genetic mutation being researched (Hwang et al., 2013). These mutations generally fall into two primary pathogenic mechanisms: deleterious loss of function (LoF) and toxic gain of function (GoF). Let's explore the various genome editing tools amenable to using zebrafish to prove the pathogenicity.

2.1.1 Gain of function studies

In zebrafish embryos, overexpression of mRNA is a common strategy to study the gain of function effects. This involves injecting an *in vitro* transcribed mRNA encoding a construct of interest into the zebrafish embryo at the 1-cell stage. This technique has been used to express patient-specific mutations in genes linked to neurological conditions like Amyotrophic Lateral Sclerosis (Armstrong and Drapeau, 2013) and small-fibre neuropathy (Eijkenboom et al., 2019) and non-neurological conditions like atrial fibrillation and sinus node dysfunction (Hoffmann et al., 2019). These studies focus on evaluating clinically significant traits like swimming behaviour, touch-evoked motor response, sensory neurite development, or electrophysiologic markers in larvae just a few days old. This makes it possible to quickly evaluate the impact of particular gene products at the early stages of development on large scales without the need for complex genetic manipulation. However, one disadvantage is the injected mRNA is only stable during the early stages (short half-

life), and the experimental variability linked to the manual microinjection of hundreds of embryos can make it challenging to interpret negative results.

To address this challenge, researchers turned to transposase-mediated stable genomic integration techniques, with Tol2 being a prominent example. The Tol2 transposon system, initially identified in Medaka, was used to create many zebrafish transgenic lines (Clark et al., 2011). The minimal Tol2 sequence is only around 200 bp long and can be easily cloned to the flanking side of the transgene of interest. For example, in many studies, the Tol2 system was employed to generate stable transgenic lines expressing the wild-type or mutant version of a gene of interest, such as the ALS-causative G348C mutation in TDP-43 (Lissouba et al., 2018)or the C1315Y mutation in *COL2A1* associated with lethal fetal skeletal dysplasia (Zhang et al., 2021). In contrast to mRNA expression studies, the Tol2 transposon system allows studying the genetic effects within stable lines. However, this transgenesis technique's main limitation is that it is random, which makes it challenging to compare wild-type and mutant allelic variants of a gene of interest as they have not been integrated at the exact genomic location and in different copy numbers.

2.1.2 Loss of function studies

In zebrafish, proving the pathogenicity of loss-of-function mutation can be less challenging, especially in the case of deleterious mutations like sudden stops or large genomic deletions, as these mutations often result in a non-functional or significantly reduced protein product. Morpholino (MO) antisense oligomers are valuable for studying loss-of-function phenotypes through transient gene knockdown. This technique involves synthetic MOs that bind to target mRNA sequences, preventing their translation into

protein. This technique has been employed for several years to stimulate gene knockdown and observe resulting phenotypic changes. For example, the morpholino technique has been used to study a variety of genes associated with different disorders, such as *CHD2* in epileptic encephalopathy (Galizia et al., 2015), *ABCC6* in *Pseudoxanthoma elasticum* (Van Gils et al., 2018), *SBDS* in Shwachman–Bodian–Diamond syndrome (Venkatasubramani and Mayer, 2008), and *VARS* in epilepsy (Siekierska et al., 2019).

However, morpholinos are injected during the early stages of embryogenesis, typically between 1-8 cell stages. Since they are resistant to degradation, they remain within cells. However, the quantity of morpholinos within each cell decreases with subsequent divisions. This makes morpholinos effective only during the first few days of development, typically up until 3 to 5 dpf. Due to this limitation, morpholinos can only be used as a transient, functional knockdown method. Moreover, recent debates have raised questions regarding the specificity of morpholinos. Stringent guidelines have been implemented for interpreting results obtained from morpholino-based assays in zebrafish. (Stainier et al., 2017). To overcome these limitations, targeted-mutagenesis assays such as Zinc Finger Nucleases (Doyon et al., 2008), Transcriptor activator-like effector nucleases (Hwang et al., 2014) or the popular CRISPR/CAS9 (Hwang et al., 2013) are widely used in many Zebrafish labs.

2.2 CRISPR-cas9 targeted mutagenesis

Inducing mutations in zebrafish genes is now relatively straightforward, thanks to the CRISPR-Cas9 genome editing technique. Using this CRISPR-cas9 technology, a wide range of mutations of interest can be studied. For loss of function mutations, the most common strategy of CRISPR-cas9 involves injecting a Cas9/and a single guide RNA

(gRNA) into the one-cell embryo (Jinek et al., 2012). This gRNA specifically binds to the targeted genomic region, and Cas9 generates a double-strand break. As the DNA ends are repaired, the repair mechanisms, particularly the Non-Homologous End Joining (NHEJ), will introduce insertions or deletions (indels) in the target locus (Brinkman et al., 2018). These indels often disrupt protein function by altering crucial amino acid sequences or causing frameshifts that result in premature stop codons and non-functional proteins (Figure 4).

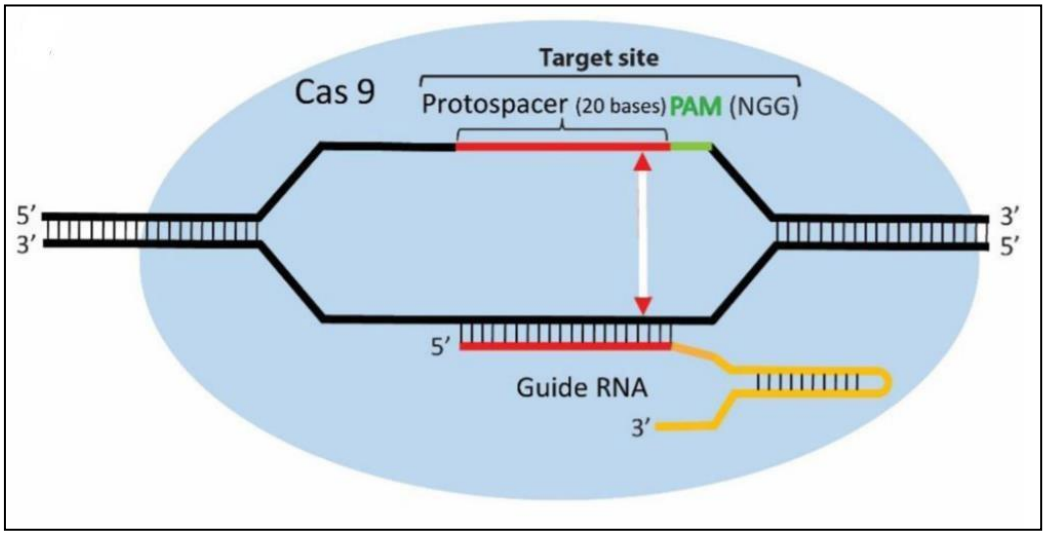

Figure 4: Mechanism of action of the CRISPR/CAS9 mutagenesis technique

Using the CRISPR-cas9 method, indels can be inserted at any region of interest. The guide RNA recognizes the target region and the CAS-9 endonucleases and cleaves the DNA. It activates the DNA repair- mechanism, which introduces indels (taken from Yamamoto & Gerbi, 2018*)*

Many loss-of-function models have been developed for various diseases like heart disease (Narumanchi et al., 2021), kidney disease (Outtandy et al., 2019), skeletal disorders and many CNS-related disorders like Amyotrophic Lateral Sclerosis (ALS) (Braems et al., 2021), Epilepsy (Samarut et al., 2018), Alzheimer's Disease (AD) (Saleem and Kannan,

2018), Parkinson's Disease (PD) (Ünal and Emekli-Alturfan, 2019), Serotonin syndrome (SS) (Stewart et al., 2013), and Glioblastoma (Reimunde et al., 2021) have been successfully modelled in the past. These studies show the potential of zebrafish to model and study the effects of loss-of-function mutation.

Moreover, utilizing a combination of multi-loci guide RNAs, targeted mutagenesis tools can also be applied for quick screening in the F0 injected larvae named "CRISPRant" (Kroll et al., 2021). The phenotypes of the injected F0 embryos or CRISPRants with mosaic loss of function (LOF) mutations can be directly examined to study the corresponding gene function (transient-knockout method).

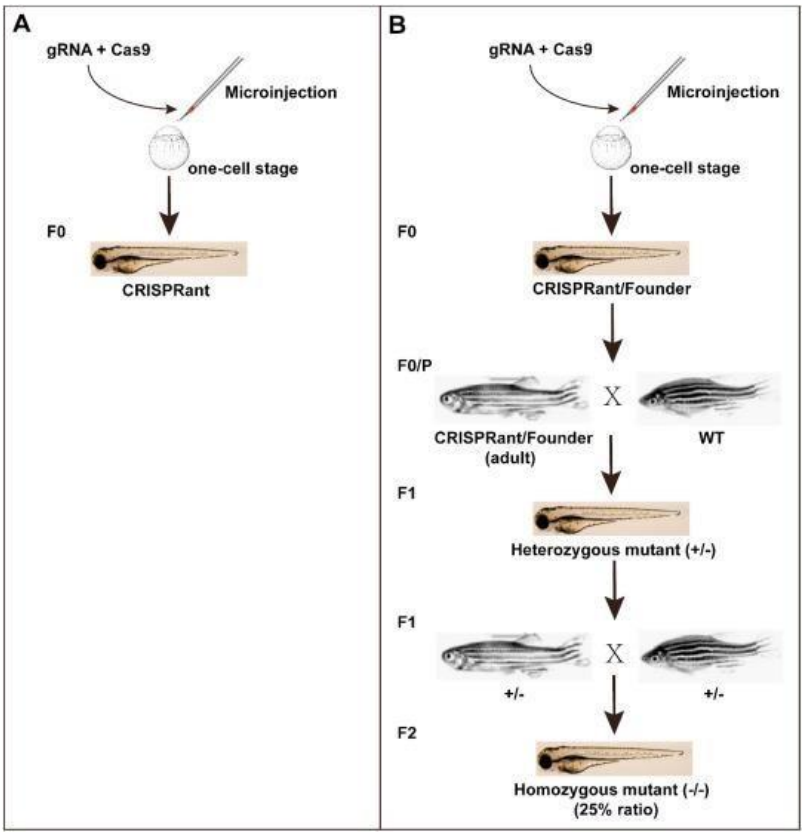

Figure 5: CRISPRant Knock out strategies *(taken from* Rouf et al., 2023)

Further, when a specific phenotype is confirmed, these injected F0 CRISPRants can be raised and further screened as founders for creating a stable mutant line (Figure 5) (Rouf et al., 2023). A recent study by Fangfang Lu et al. utilized the F0 CRISPRant technique to model approximately 27 candidate genes associated with retinal pigment epithelium (RPE) regeneration. Using these F0 CRISPRants, they screened for pro-regenerative genes and identified positive and negative regulators of RPE regeneration. Interestingly, one candidate gene showed novel roles in regulating macrophage infiltration and debris clearance during RPE regeneration (Lu et al., 2023). This study shows the potential of using F0 CRISPRant to investigate the function of a gene of interest quickly.

Further CRISPR-cas9 mutagenesis can also be employed to mimic patient-specific missense mutations onto the endogenous zebrafish gene. Using the Homology-mediated repair strategy, small modifications such as single nucleotide editing or LoxP integration can be achieved by adding a nucleic acid donor template to the CRISPR cocktail to be microinjected (Chang et al., 2013). By using this approach, Armstrong et al. (2016) successfully generated a zebrafish model of amyotrophic lateral sclerosis (ALS) by inserting two SNPs in the zebrafish *tardbp* and *fus* genes (tardbpA379T and fusR536H, respectively). These SNPs correspond to the tardbpA382T and fusR521H point mutations identified in patients with ALS (Armstrong et al., 2016). Also, patient-specific genetic avatars were successfully modelled in *FBN1* in various heritable connective tissue disorders (HCTD) (Yin et al., 2021), *RPS14* in myelodysplastic syndrome (MDS) (Ear et al., 2016). However, due to technical limitations, this knock-in application's overall efficiency remains much lower than the generation of random knockout mutations. Another limitation of this technique is that the specific protein residue affected by the mutation

might not be the same in zebrafish. Also, even if an orthologue gene exists in the zebrafish genome, the specific missense mutations may not have the same impact on human or fish proteins (Ochenkowska et al., 2022).

Ultimately, zebrafish serve as an attractive multi-assay proxy to characterize the pathogenicity of genetic mutations, as shown in Figure 6. Despite specific technical challenges that might hinder the standardization of these functional approaches *in vivo*, zebrafish functional characterization can provide additional biological evidence to bridge the gap between variant identification and their pathogenic classification.

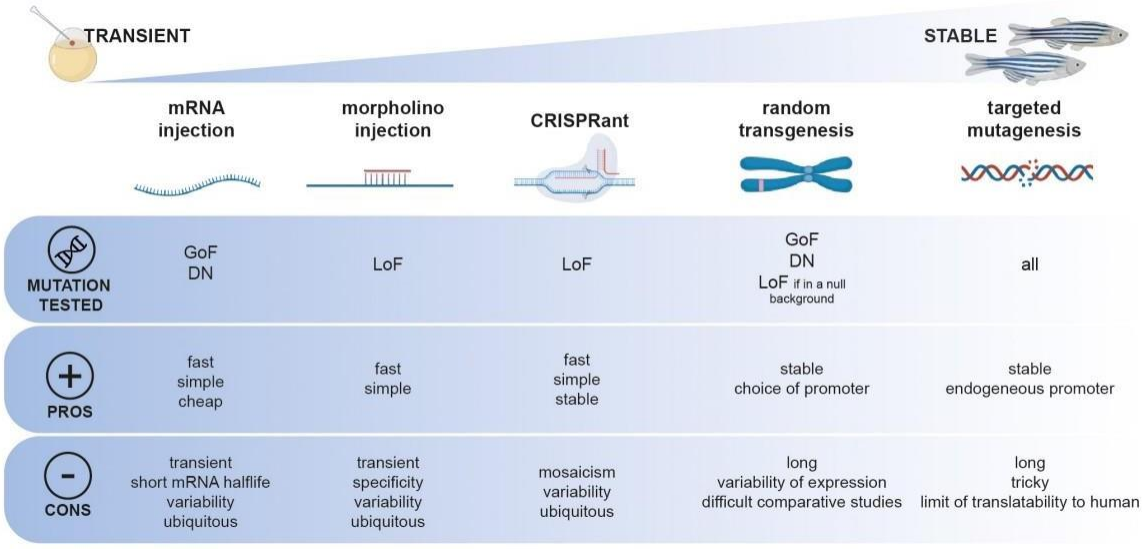

Figure 6: Functional toolbox to study the function of genetic mutation in zebrafish. *(taken from* Ochenkowska et al., 2022*)*

Finally, in this section, we have described the use of CRISPRant to evaluate loss-of-function mutations in zebrafish functionally. I will employ this technique in my research project to investigate the effects of the gene "*INTS11*," which will be discussed in the following section.

3. The Integrator Complex

The Integrator complex (INT) is a multi-subunit protein assembly first identified in 2005 by researchers investigating the complex machinery involved in gene expression and RNA processing (Baillat et al., 2005). The Integrator complex consists of at least 15 protein subunits (*INTS1-INTS15*), which exhibit phylogenetic conservation among metazoans (Beckedorff et al., 2020).

The Integrator complex was initially recognized as a vital component responsible for the cleavage of the 3'-ends of small nuclear RNAs (snRNAs), which are critical components of the cellular splicing machinery (Baillat et al., 2005). This cleavage step is important for snRNAs' maturation, stability, and functionality, which play crucial roles in pre-mRNA splicing. Moreover, the role of the Integrator complex has further expanded to encompass the processing of several non-coding RNAs such as long noncoding RNAs (lncRNA) (Nojima et al., 2018), several Herpes virus transcripts (Cazalla et al., 2011), enhancer RNAs (eRNAs) (Lai et al., 2015), telomerase RNA (tertRNA) (Rubtsova et al., 2019), and messenger RNAs (mRNAs). The Integrator complex has recently gained attention for its role in regulating the transcription of coding mRNAs. It acts as a global regulator of RNA Polymerase II (RNAPII) activity, impacting various aspects of gene expression, including transcriptional activation, RNAPII pause release, and transcription termination (Gardini et al., 2014; Vervoort et al., 2021; Huang et al., 2020; Skaar et al., 2015). Overall, the Integrator complex is vital in the broader context of cellular function and molecular biology by participating in various RNA processing pathways and regulating gene expression.

3.1 Various subunits of the Integrator complex

The Integrator complex is composed of several subunits from *INTS1* to *INTS15*. Each subunit forms distinct functional modules, which play a crucial role in the overall function of the Integrator complex. The various modular components of the Integrator complex are the enhancer module, phosphatase module, endonuclease module, central backbone, and Sensor of Single-Stranded DNA (SOSS) Complex (Figure 7).

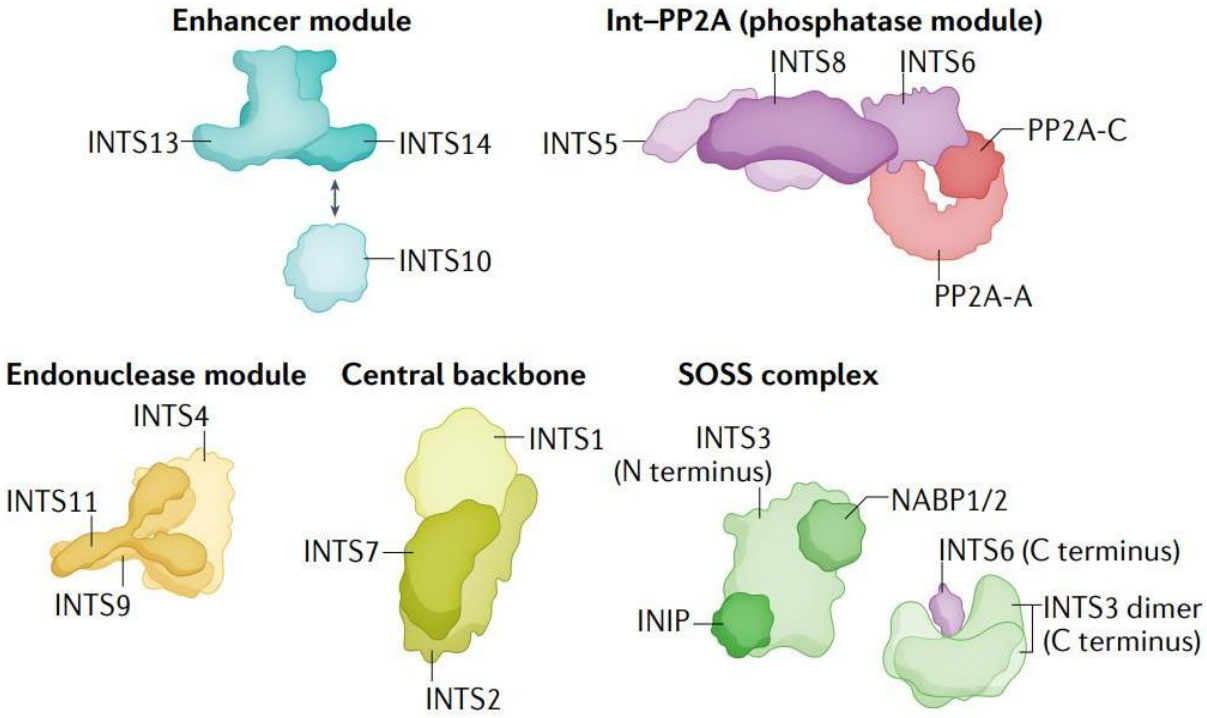

Figure 7: Integrator subunits and their modular components. Please note that the *INTS11* subunit studied in this research project is part of the endonuclease module. *(taken from* Welsh and Gardini, 2023*)*

3.1.1 Enhancer Module

This module includes *INTS13* and *INTS14*, which form a stabilizing heterodimer. Additionally, *INTS10* associates with the *INTS13-INTS14* heterodimer, although structural

information about *INTS10* is currently unavailable. This module plays a vital role in maintaining the stability of the Integrator complex.

3.1.2 Integrator-Serine/Threonine-Protein Phosphatase 2A Module (Int-PP2A)

This module regulates protein phosphorylation, influencing RNA processing and cellular functions. It includes the PP2A heterodimer, composed of two subunits, PP2A-A and PP2A-C. It assembles on *INTS5* and *INTS8*, with *INTS6* acting as a bridge to *INTS8* (Figure 7) (Baillat and Wagner, 2015).

3.1.3 Endonuclease Module

This module consists of *INTS11* (catalytic subunit), *INTS9* (catalytic, inactive), and *INTS4* (scaffold subunit). The endonuclease module is responsible for the precise cleavage of RNA molecules, essential for various cellular processes (Pfleiderer and Galej, 2021).

3.1.4 Central backbone

The Central Backbone of the Integrator complex is composed of *INTS1*, *INTS7*, and *INTS2*. It provides structural stability and a scaffold for other functional modules within the complex.

3.1.5 Sensor of Single-Stranded DNA (SOSS) Complex

The Sensor of Single-Stranded DNA (SOSS) Complex, centred around *INTS3*, plays a critical role in DNA repair mechanisms. While the complete structure of SOSS Complex is not fully known, *INTS3* interacts with various subunits of the SOSS complex, such as NABP1, NABP2, and INIP.

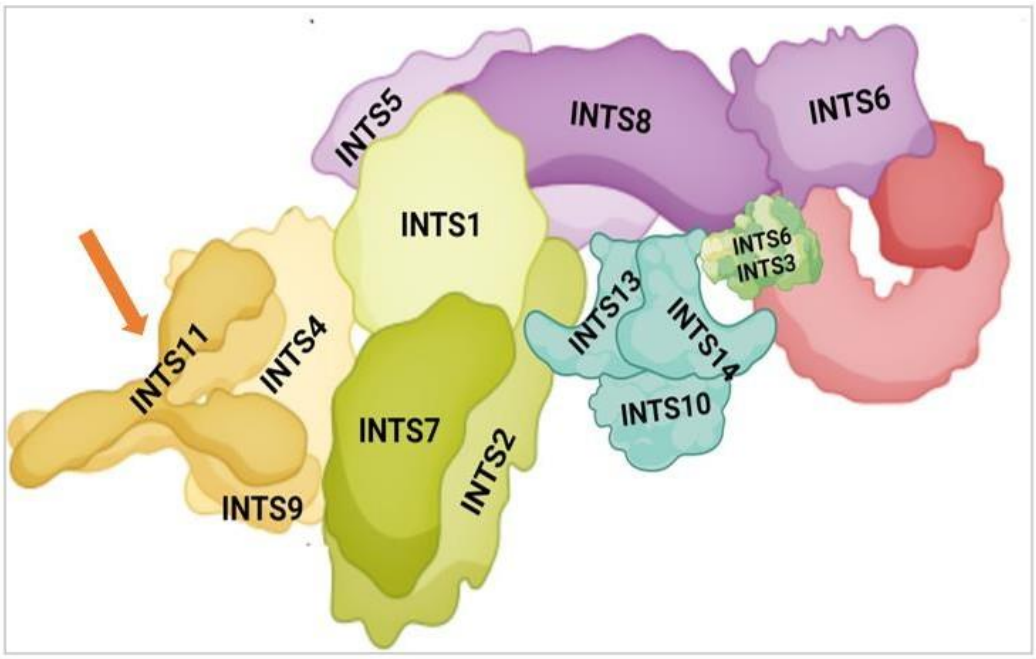

Figure 8: Structure of the Integrator complex

Subunits of the integrator complex. INTS11, the gene of interest in this study, is highlighted with an orange arrow

(adapted from Welsh and Gardini, 2023, *using Biorender)*

Altogether, the individual role of each subunit is essential for the proper functioning of the Integrator complex (Figure 8). Specifically, *INTS11*, the gene of interest in this thesis, will be discussed in more detail in the following section.

4. Introduction to the *INTS11* Gene

The *INTS11* (Integrator complex subunit-11) gene encodes a protein that is crucial to the catalytic core of the Integrator complex. It was initially identified by Baillat et al. in 2005 (Baillat et al., 2005). In humans, the functional protein consists of 600 amino acids (Wu et al., 2017). *INTS11* is also known by various aliases, such as CPSF3L, CPSF73L, FLJ20542, *INT11*, and RC-68.

The endonuclease activity of the Integrator complex is mediated by its subunit 11 (*INTS11*), which contains a metallo-β-lactamase domain and β-CASP domain, Zn-dependent metallo hydrolase RNA specificity domain (Figure 9) (Kirstein et al., 2021).

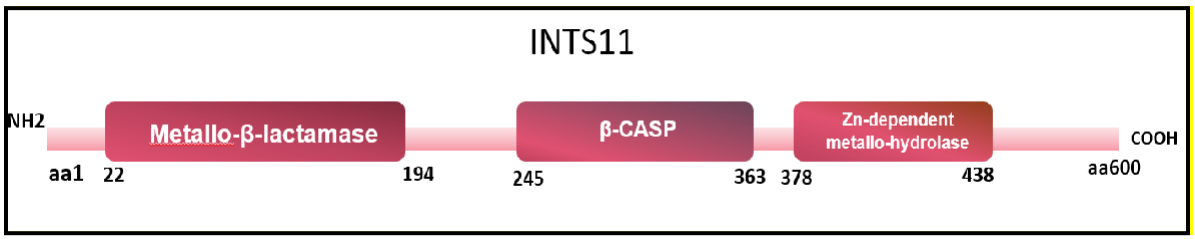

Figure 9: Functional domains of *INTS11*

INTS11 forms a heterotrimer with *INTS4* and *INTS9* to create an ‘‘Integrator Cleavage Module,” which is important for the function of the Integrator complex. *INTS11* establishes a stable complex with Integrator complex subunit 9 (*INTS9*) through its C-terminal domain (CTDs). Unlike most subunits of the Integrator complex, *INTS11* and *INTS9* share nearly identical functional domains and are paralogs of the cleavage and polyadenylation specificity factor subunits CPSF73 and CPSF100, respectively (Baillat and Wagner, 2015).

The Integrator cleavage module facilitated by *INTS11* plays a pivotal role in cleaving the 3' ends of various noncoding RNAs, snRNAs, and nascent mRNAs of protein-coding genes. Thus, this catalytic activity is a key functional component of Integrator-mediated transcriptional repression and biogenesis of non-coding RNA (Rienzo and Casamassimi, 2016; Pfleiderer and Galej, 2021).

4.1 Role of *INTS11* gene in snRNA processing

Small nuclear RNAs are often called "uridine-rich small nuclear RNAs" (U snRNAs) due to their high uridine content. These non-coding RNAs, typically ranging from 60 to 200 nucleotides in length, are found across all eukaryotic cells. They lack are not polyadenylated and exhibit high expression levels.

The major snRNAs, including U1, U2, U4, U5, and U6, are abundant and serve as general components of the splicing machinery. They are involved in splicing common U2-type introns, which are the majority of introns in eukaryotic genomes. While the minor snRNAs, namely U11, U12, U4atac, and U6atac, are specialized for handling a specific subset of introns. Most major and minor U snRNAs are transcribed by RNA polymerase II (RNAPII). However, U6 snRNA is transcribed by RNA polymerase III (RNAPIII). These snRNAs undergo a complex maturation process facilitated by the Integrator complex (Matera et al., 2007; Marzluff et al., 2008).

INTS11 plays a crucial role in this process by cleaving the 3'-ends of nascent snRNA transcripts, which leads to the termination of RNA polymerase II (RNAPII) transcription. The cleaved snRNA transcripts are transported to the cytoplasm for further 3' trimming and association with proteins to form small nuclear ribonucleoproteins (snRNPs). These snRNPs, including Sm proteins, are vital components of the spliceosome that play a pivotal role in pre-mRNA splicing, a fundamental process involving the removal of introns and retaining exons within mRNA molecules (Figure 10). This splicing process is essential for regulating gene expression and protein synthesis (Matera and Wang, 2014; Karijolich and Yu, 2010; Mendoza-Figueroa et al., 2020).

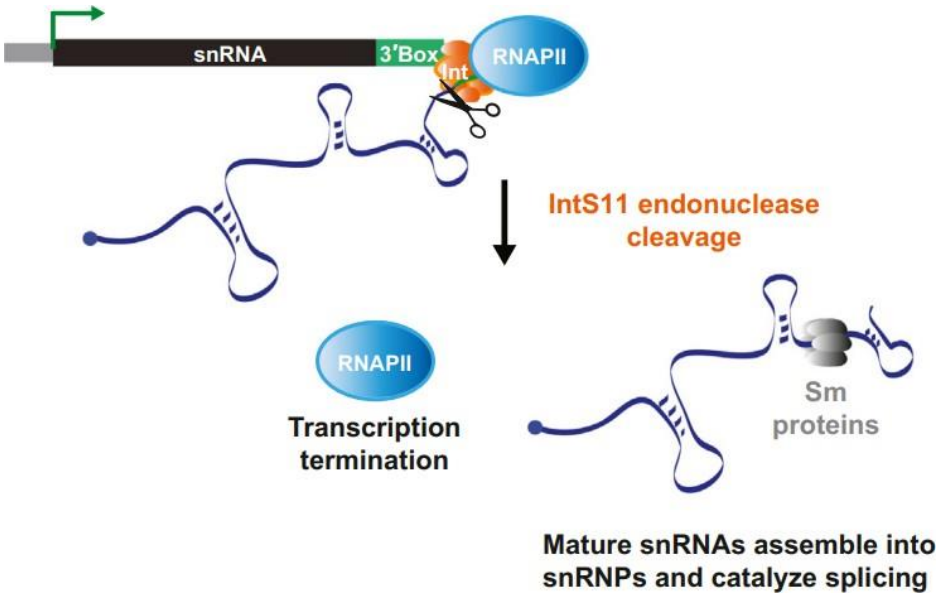

Figure 10: Role of *INTS11* gene in snRNA processing

Nascent snRNA transcripts are cleaved upstream of the 3'-box sequence by INTS11, leading to the termination of RNA polymerase II (RNAPII) transcription. The cleaved snRNA transcript is released, further processed, and assembled into an RNA–protein complex (snRNP), which includes Sm proteins that catalyze pre-mRNA splicing reactions

(taken from Mendoza-Figueroa et al., 2020)

Moreover, studies have consistently demonstrated that depletion or mutation in *INTS11* results in a significant accumulation of unprocessed small nuclear RNAs (snRNAs). Failure to process snRNAs can lead to longer transcripts with heterogeneous 3' ends. These transcripts can arise through various mechanisms, including transcription termination, ectopic processing events defining specific 3' ends, and precocious polyadenylation (Ezzeddine et al., 2011).

In human cells, the depletion of *INTS11* using RNAi techniques leads to a substantial increase in primary snRNA transcripts. This indicates that *INTS11* is crucial in

processing the 3' ends of U1 and U2 snRNA transcripts (Baillat et al., 2005; O'Reilly et al., 2014). Similarly, in *Drosophila*, the knockdown of INTS11 proteins results in an increased accumulation of misprocessed spliceosomal snRNAs (Ezzeddine et al., 2011). In *C. elegans*, *INTS11* disruption leads to aberrant snRNA processing, subsequently affecting the transcription patterns of genes downstream of snRNA loci (Gómez-Orte et al., 2019). A recent study has shown that disruptions in other subunits of the Integrator complex in flatworm *Schmidtea mediterranea* can also result in unprocessed snRNAs. Importantly, these disruptions significantly impact global mRNA splicing, leading to widespread alterations in the splicing patterns of mRNA transcripts (Schmidt et al., 2018).

Altogether, these studies underscore the essential role of *INTS11* in accurately cleaving and processing the 3' ends of the U snRNAs. The crucial role of the *INTS11* gene in snRNA processing is paramount for maintaining accurate gene expression by ensuring the proper assembly and function of essential RNA components (Chen and Wagner, 2010).

4.2 Role of Integrator in developmental disorders and cancer

The Integrator complex plays a crucial role in all tissue types and developmental stages by controlling transcription and RNA processing in metazoans. The consequences of disrupting the integrity or function of the integrator complex might range from developmental issues to cancer, depending on which subunit has been affected (Welsh and Gardini, 2023).

4.2.1 Developmental defects associated with the Integrator complex

Integrator function is necessary for early development, tissue morphogenesis and cell differentiation in the adult organism. For example, deleting the *INTS1* subunit in

developing mouse embryos leads to premature lethality, likely by destabilizing the entire complex (Hata and Nakayama, 2007). In *Drosophila melanogaster*, mutations in the core components of the Integrator complex have resulted in lethality during the mid-to-late larval stage (Ezzeddine et al., 2011). Notably, mutations in *INTS6* in zebrafish embryos hinder gastrulation, leading to significant dorsalization. This underlines *INTS6's* role in embryonic patterning (Kapp et al., 2013). The catalytic subunit *INTS11* is crucially required in early embryonic stages in *Artemia sinica* (brine shrimp), as emphasized by the observed delay in embryonic development upon *INTS11* knockdown (Huang et al., 2020).

Moreover, a fully functional Integrator complex is essential in coordinating transcriptional programs during later developmental stages and in differentiated adult stem cells. In the stem cell-rich planarian flatworm, the absence of core Integrator subunits disrupts stem cell maintenance and tissue regeneration (Schmidt et al., 2018). Similarly, in a mouse adipogenesis model, the differentiation process depends on the elevated expression of Integrator subunits (Otani et al., 2013). A study by Debbie et al. demonstrates that the Integrator complex controls the expression of genes crucial for the migration of newly generated neurons across the developing cerebral cortex in mouse neural progenitor cells (van den Berg et al., 2017).

Although all subunits of the integrator complex are crucial, each subunit has a unique role. Moreover, a specific subunit's loss or improper functioning can lead to a particular phenotype. This implies that even though they are part of the same complex, each subunit may have distinct molecular characteristics.

4.2.2 Integrator complex mutations in cancer

Additionally, mutations in the Integrator complex have been implicated in numerous cancer cases (Federico et al., 2017). Particularly, reduced expression levels of several Integrator subunits correlate with reduced overall survival rates in diverse cancer cohort studies (Barra et al., 2020). In acute myeloid leukaemia (blood and bone marrow cancer), missplicing occurs in *INTS3*. This missplicing disrupts the proper functioning of *INTS3*, which, in turn, affects the expression and activity of other subunits within the Integrator complex. Due to this disruption, the cells lose their ability to differentiate or mature properly, a critical process for normal development and tissue function (Yoshimi et al., 2019).

4.3 Human mutations in Integrator complex subunits

Although the integrator complex comprises 15 subunits, only three subunits (*INTS1, INTS8, INTS13*) have been linked to human disease, as shown in Table 1. The detailed phenotypes are discussed below.

Subunit	Molecular function	Main phenotype
INTS1 (biallelic)	Central backbone, provides scaffolding to catalytic modules	Cognitive delay, absence of speech, cataracts and/or glaucoma, facial dysmorphism
INTS8 (biallelic)	Phosphatase module, required for PP2A recruitment	Cognitive delay, absence of speech, motor impairment
INTS13	Enhancer module, not required for cleavage or phosphatase activity	Oral–facial–digital anomalies, speech abnormality

Table 1: Human mutations in Integrator complex subunits

(taken from Welsh and Gardini, 2023*)*

Individuals with biallelic mutations in *INTS1* exhibit a range of developmental issues, including limited or absent speech, abnormal gait, cataracts, intellectual disability, and craniofacial anomalies. These findings are further supported by a mutant zebrafish model displaying abnormal eye development similar to cataracts identified in human patients (Oegema et al., 2017; Krall et al., 2019; Zhang et al., 2020).

Additionally, recessive mutations in the *INTS8* gene lead to a severe neurodevelopmental disorder characterized by brain structural anomalies, facial and limb dysmorphism, intellectual disability and epilepsy. The fibroblasts derived from patients with these mutations show disruptions in RNA processing and gene expression, indicating abnormalities in mRNA transcription and processing (Oegema et al., 2017).

More recently, *INTS13* has been associated with an autosomal recessive developmental ciliopathy characterized by Oral-Facial-Digital anomalies and speech abnormalities. Depletion of *INTS13* in human cultured cells disrupts ciliogenesis and triggers the dysregulation of a broad spectrum of ciliary genes (Mascibroda et al., 2020).

Overall, these studies highlight the significance of the Integrator complex in maintaining proper neurodevelopmental processes. They also suggest that the brain may be particularly susceptible to disruptions in Integrator activity. However, further research is required to fully elucidate the roles of each subunit and the implications of their mutations.

CHAPTER:2 PROJECT

1. Background

We recently collaborated with Dr. Reza Maroofian, a geneticist at University College London, who identified multiple families carrying loss-of-function mutations in the *INTS11* gene, the catalytic component of the Integrator complex. Biallelic mutations in *INTS11* are associated with profound neurodevelopmental delays, intellectual disabilities, impaired motor development, and brain atrophy. More comprehensive information regarding this phenotype is discussed later in this section.

While other subunits in the Integrator complex (*INTS1*, *INTS8*, and *INTS13*) have been associated with neurodevelopmental-related issues, no prior studies have reported *INTS11* mutations in humans in the literature. Additionally, there was a lack of conclusive functional evidence linking *INTS11* mutations to neurodevelopmental disorders.

Note: Before we began this project, INTS11 mutations had not been associated with any diseases in patients. However, during the final stages of this project, a study reported biallelic mutations in INTS11 patients with neurodevelopmental problems (Tepe et al., 2023). *Nevertheless, the functional characterization of INTS11 loss-of-function in this study is limited, lacking validation at the level of snRNA processing and assessing the consequences at the central nervous system level in vivo.*

In this study, we investigated the impact of *ints11* loss-of-function in zebrafish, which is an excellent model for studying neurodevelopment. Zebrafish have a single copy

of the *ints11* gene (ENSDARG00000025212), and the encoded protein is 84% identical to the human INTS11 protein. Moreover, the functional domains are conserved between human and zebrafish proteins (Figure 11), establishing it as a relevant model for studying the *INTS11* gene.

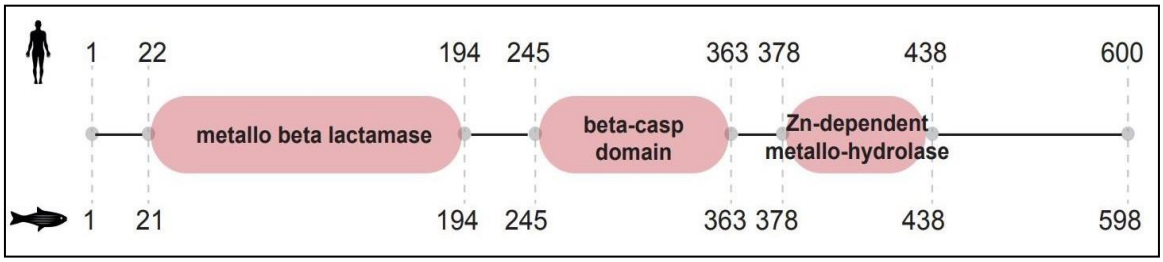

Figure 11: Functional domains of *INTS11* gene in humans and zebrafish.

2. Objective

The main objective of this research project is to investigate the effect of *ints11* loss-of-function *in vivo* in zebrafish, particularly at the central nervous system (CNS) level. This will allow inferring a pathogenic role to these mutations regarding the clinical presentations of patients carrying biallelic mutations in *INTS11*.

3. Clinical data from patients

The patient's clinical information discussed in this section was collected from our collaborator, Dr. Reza Maroofian. Variants in *INTS11* were identified through whole-exome sequencing performed on DNA samples obtained from the probands. There were 7 families exhibiting *INTS11*-loss-of-function mutations, as shown in the pedigree (Figure 12).

All variants were reported according to standardized terminology defined by the reference human genome GRCh38 (hg38), specifically for the GenBank transcript NM_017871.6 and protein sequence NP_060341.2.

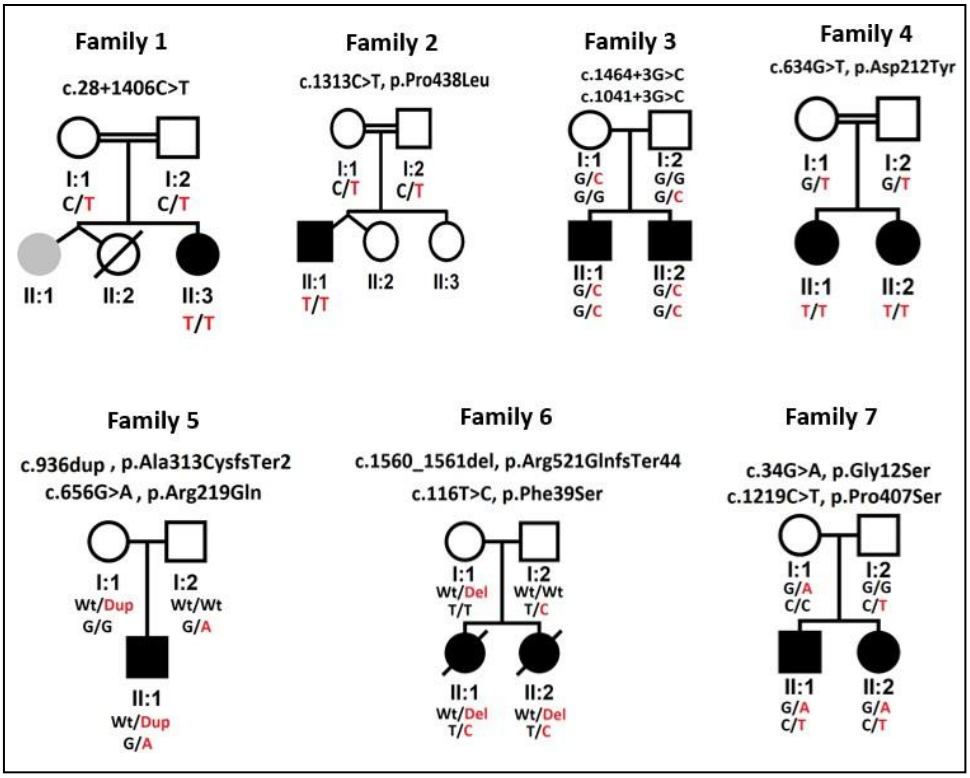

Figure 12: Pedigree of affected families with *INTS11* loss of function mutation

Individuals with *INTS11* mutations exhibit a wide range of neurological abnormalities (Table 2). Most probands have developmental difficulties, including global developmental delay, intellectual disability, speech impairment, and motor developmental abnormalities.

	Family 1	Family 2	Family 3		Family 4		Family 5	Family 6		Family 7	
	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7	Subject 8	Subject 9	Subject 10	Subject 11
Sex	female	male	male	male	female	female	male	female	female	male	female
Age	5 years	4 years	18 years	6 years	11 years	8 years	2 years	18 years (deceased)	6 years (deceased)	18 years	17 years
Coding change	c.28+1406C>T	c.1313C>T	c.1041+3G>C	c.1464+G>C	c.634G>T	c.634G>T	c.936dup;c.656G>A	c.1560_1561del	c.116T>C	c.34G>A	c.1219C>T
Protein change	NA	p.Pro438Leu	NA	NA	p.Asp212Tyr	p.Asp212Tyr	p.Ala313CysfsTer2, p.Arg219Gln	p.Arg.GlnfsTer44	p.Phe39Ser	p.Gly12Ser	p.Pro407Ser
Global developmental delay	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Intellectual disability	+++	+	+++	+	++	++	+++	+++	+++	+++	+++
Language delay	+++	++	+++	++	++	+++	+++	+++	+++	++	++
Motor development and disorders	+	+	+++	+++	+++	+++	+++	+++	+++	+	+
Brain MRI	cerebellar hypoplasia, abnormal appearance of the posterior fossa	cerebellar atrophy, thin corpus callosum	cerebellar and basal ganglia atrophy, severe hypomyelinatio	mildly dilated 4th ventricle and suspected mild atrophy of cerebellum	cerebellar atrophy subcortical midfrontal lobe lesion	mild to moderate cerebral atrophy	progressive supratentorial and infratentorial atrophy, leukoencephalopath	progressive cerebral atrophy, gyral simplification, pontocerebellar atrophy	cerebral progressive hypolapsia	(at age 2 years) small cerebellum; delayed myelination	(at age 9 years) progressive cerebellar atrophy; diffuse white matter signal changes
Behavioural symptoms	Autistic hyeractive	Autistic hyeractive	na	Repetitive hand movements	no	no	no	na	na	Repetitive hand movements	Autistic hyperactive
Ataxia	Trunk atxia	Limb ataia	no	no	Limb	Gait ataxia	no	na	na	Trunk ataxia	Gait ataxia
Hypotonia	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Seizures	–	–	yes	yes	yes	yes	yes	yes	yes	–	one
Microcephaly	–	–	yes	yes	yes	yes	yes	yes	yes	yes	–
Optic findings	strabismus, myopia	mild optic atrophy	myopia	optic atrophy	na	mild optic atrophy	optic atrophy	optic atrophy	optic atrophy	retinal dystrophy	hypermetropia

Table 2: Variants in *INTS11* and their clinical phenotype

Magnetic resonance imaging (MRI) of the brain revealed distinct alterations, including atrophy in both the cerebellar and basal ganglia regions, as well as progressive

cerebellar hypoplasia, gyral simplification, smaller cerebellum, leukoencephalopathy, dilated ventricles, and thin corpus callosum. Interestingly, certain patients display hyperactivity alongside symptoms reminiscent of autism spectrum behaviours. Electroencephalogram (EEG) abnormalities have also been recorded, with a subset of patients presenting epileptic episodes and seizures. Notably, ataxic problems in the limbs, gait, and trunk have been reported in some patients. Furthermore, optic atrophy and visual impairment was a prevalent feature in most subjects. It is also important to note that most variants identified in the study are not clustered in specific domains, as shown in Figure 13.

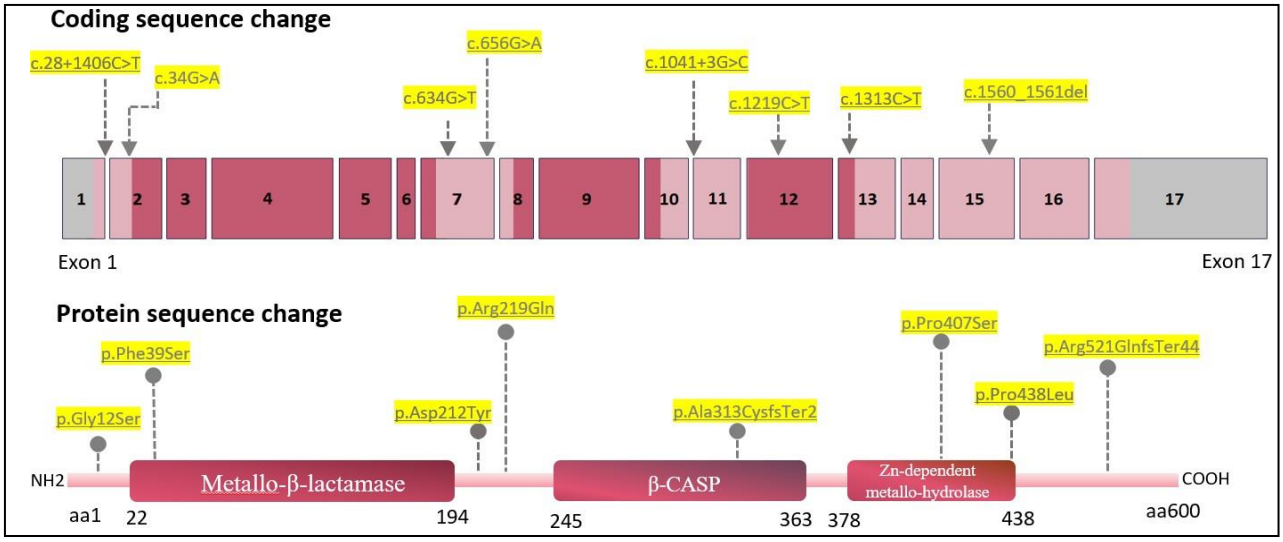

Figure 13: A schematic presentation of the variants at the level of *ints11* transcript (top) and INTS11 protein (bottom)

4. Materials and Methods

4.1 Zebrafish husbandry

Adult zebrafish (*Danio rerio*) were maintained at a temperature of 28.5 °C under a 12/12-hour light/dark cycle, following the staging protocol previously described (Kimmel et al., 1995). All animal experiments were conducted in accordance with the guidelines of the Canadian Council for Animal Care at the Research Center of the University of Montreal Hospital Center.

4.2 sgRNA and Cas9 preparation and microinjection

We utilized the CRISPR/Cas9 technology to generate *ints11* F0 knockouts (CRISPRants) in zebrafish. Specifically, we designed 3 single-guide RNAs (gRNAs) targeting the *ints11* gene using the online tool CRISPR scan. The PAM (protospacer adjacent motif) sequence is indicated in parenthesis.

- Exon 4: CAGGATGGGGCAGATGGCTT(TGG),
- Exon 9: CCCGGTGGAGAAATAAATGG(GGG),
- Exon 13: AGCGCTCACGCAGACGCCAA(AGG).

As controls, we designed guide RNAs with the same sequence but introduced 5 mismatches in the 3' part of their gene-specific sequence. The mismatch sequences are shown in lowercase and highlighted in red:

- Exon 4: CAGGATGGGGCcGgTtaCcT,
- Exon 9: CCCGGTGGAGAgAcAtAgGt,
- Exon 13: AGCGCTCACGCcGgCtCtAg.

We synthesized gRNA and Cas9 mRNA as described previously (Samarut et al., 2016). A mixture of solution containing the Cas9 mRNA (100 ng/µl) and gRNAs (30 ng/µl) was injected into one-cell stage zebrafish embryos using a Picospritzer IV pressure ejector.

4.3 High-resolution melting

For each assay, we confirmed the mutagenic potential of our gRNAs and verified the non-mutagenicity of 5-mismatch gRNAs by high-resolution melting genotyping (HRM), as detailed in Samarut et al., 2016. HRM primers were designed using the APE software version 2.0.70.0 (Davis and Jorgensen, 2022)

- Exon 4

  Forward: ACCGTGATCTTCCTGAAGTC

  Reverse: CGAGATGGTGGGTTACGACG

- Exon 9

  Forward: TAGTGATTGGCCTTCTCCGTC

  Reverse: GTTGTATAATTTCAGCCTGTTTCATG

- Exon 13

  Forward: CTGCCATGCGGATCAACTGC

  Reverse: TTTGTGTGTGTTCAGTTGGATG

The PCR reactions were made with 2.5 µL of the Precision Melt Supermix for HRM analysis (Bio-Rad #172-5112), 0.5 µL of each primer (10 µM) and 1 µL of genomic DNA and distilled water to reach a total volume of 5µL. The PCR reaction was performed using a LightCycler 480 Instrument II (Roche) with either white 96 well plates or 8 PCR strips, depending on the number of samples. The Two-step Evagreen PCR protocol involves an initial 2-minute heating at 95°C, followed by 45 cycles of temperature changes: 95°C for

10 seconds and 60°C for 30 seconds. The reaction concludes with a gradual cooling from 95°C to 10°C at a rate of 0.02°C per second, followed by maintaining a stable temperature at 40°C. Melting curves were analyzed using the LightCycler 96 software version 1.1 (Roche).

4.4 qPCR primer design for unprocessed snRNAs

As the primary role of the *INTS11* gene is snRNA processing, we aimed to validate our F0 CRISPR knockout model for unprocessed snRNAs. For our analysis, we used an unpublished gene expression dataset from our laboratory to identify the top 6 highly expressed snRNAs in zebrafish larval brains. Subsequently, we designed specific primers for amplifying the 3' unprocessed regions of these snRNAs using APE software version 2.0.70.0 (Davis and Jorgensen, 2022). The primer sequences are as follows:

snRNA	Forward primer	Reverse primer
U1_4snRNA	AAATGTGGGAATCTCGACTGCATG	CTCGTGTGTCCTTGATTGTGTGTG
U2_snRNA	GCATCGACCCGGTATTGCAG	ACGAACCAATCTCCACATGC
U2_1snRNA	GCATCGACCCGGTATTGCAG	TCGTTGATACACATCATTCG
U2_2snRNA	GCATCGACCCGGTATTGCAG	ATGTATCAATCCGTCTTATCTC
U5_snRNA	TGATGCCCTGCCTATCGGTG	AGGTTCCATCCGTTATTTCTCTTTC
U12_snRNA	TTTGAACGGGTACAGGTCTGC	TTTAACCTGTTATTGGGTGTTGTCG

Table 3: qPCR primer sequence for small nuclear RNAs

4.5 RT-qPCR for unprocessed snRNAs

The total RNA was extracted from 5 dpf *ints11* whole larvae using the picopure RNA extraction kit (Thermo Fisher Scientific) following the manufacturer's standard protocol. For each sample, RNA was extracted from a pool of 5 whole larvae, microdissected brains or trunks. The samples were further evaluated by nanodrop spectrophotometry, accessing the 260/280 and 260/230 ratios to check for any contamination with chemicals. Reverse transcription was performed from 500ng of total RNA using the Superscript VILO reverse transcription mix (Invitrogen). This step converted the RNA to cDNA, which was used as the template for the subsequent qPCR analysis. Quantitative PCR was performed on 2 µL of 1:10-diluted cDNA using SYBR Green I master (Roche) on a LightCycler 80 thermocycler.

4.6 Phenotypic characterization

The morphological analysis was performed on 5 days post fertilization (dpf) animals. Larvae were immobilized in a 3% methylcellulose cavity, and images were taken using a stereomicroscope (Leica S6E). Body length, head and eye sizes were measured in millimetres (mm) from scale-calibrated images using ImageJ software (National Institutes of Health, Bethesda, Maryland).

4.7 Behaviour assay

We monitored the swimming behaviour of 5 dpf- old larvae during a 1-hour dark followed by a 1-hour light period using DanioVision (Noldus Wageningen, The Netherlands). 5 dpf larvae were separated into single wells of a 96-well plate containing 200 µL of E3 media and habituated in the Daniovision (Noldus Wageningen, The

Netherlands) recording chamber for 1 hour before the start of the experiment. Ethovision XT12 (Noldus) software was used for analyzing the distance swam (in millimetres) and maximum acceleration during the 1-hour dark and 1-hour light periods.

4.8 Survival assay

We observed the survival of *ints11* larvae and controls over 15 days in a 500ml beaker. The larvae were fed twice daily with a dry powdered brine shrimp diet, and any deceased larvae were removed each morning. We calculated the survival rates using the Kaplan-Meier survival curve analysis.

4.9 Immunostaining and cerebellum quantification

Immunostaining against the anti-parvalbumin-7 (PAV7) antibody was performed to check the integrity of the cerebellum in *ints11* larvae. The larvae were fixed in 4% paraformaldehyde in PBS (Phosphate-Buffered Saline). After rehydrating gradually into PBST (Phosphate-Buffered Saline with Tween 20), the larvae were permeabilized for 10 min in cold acetone.

Larvae were then blocked and permeabilized with a solution containing 5% normal goat serum, 1% BSA (Bovine Serum Albumin) and 1% DMSO (Dimethyl Sulfoxide) diluted in 1X PBS for 1.5 hours at room temperature. Subsequently, they were incubated overnight at 4°C with an anti-Parvalbumin7 antibody (1:1000, mouse monoclonal). After several washes, larvae were incubated with the goat anti-mouse secondary antibody coupled to Alexa Fluor 488 overnight. Following additional washes, fluorescence was analyzed using confocal microscopy (fixed-stage Olympus microscope BX61WI). Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, Maryland).

4.10 Statistical analysis

All statistical analyses were performed using Prism 9 (GraphPad Software, Version 9). When comparing the two groups, we employed Student's t-test. For datasets involving more than two groups, we utilized one-way and two-way analysis of variance (ANOVA) as appropriate. In survival analysis, we employed the Kaplan-Meier survival curve method to assess and visualize survival data. Additionally, Pearson correlation coefficients were calculated to examine relationships between mutagenic scores of *ints11* guide RNAs and the behavioural phenotype.

5. Results

5.1 Generation of F0 knockouts in zebrafish using CRISPR/Cas9

In this project, we chose to generate F0 CRISPRants of the *ints11* gene, as it was more convenient and quicker compared to the traditional approach of establishing a stable zebrafish line (Kroll et al., 2021). As a first step, we designed CRISPR guide RNAs targeting three exons that encode important protein functional domains, namely exons 4, 9, and 13. Subsequently, we injected a cocktail containing all three CRISPR guide RNAs along with cas9 mRNA into single-cell zebrafish embryos. As controls, we used embryos injected with the same mutagenic molecular cocktail but with 5-mismatch control gRNAs, as described previously in the methods. Mismatch controls are preferred over uninjected controls because they are specific to the mutagenic potential of the guide RNAs without introducing potential variability that may arise from injecting three gRNAs and Cas9 mRNA. We validated the cutting efficiency of all our guide RNAs targeting *ints11*, as well as mismatch controls, using high-resolution melting assay (HRM) genotyping.

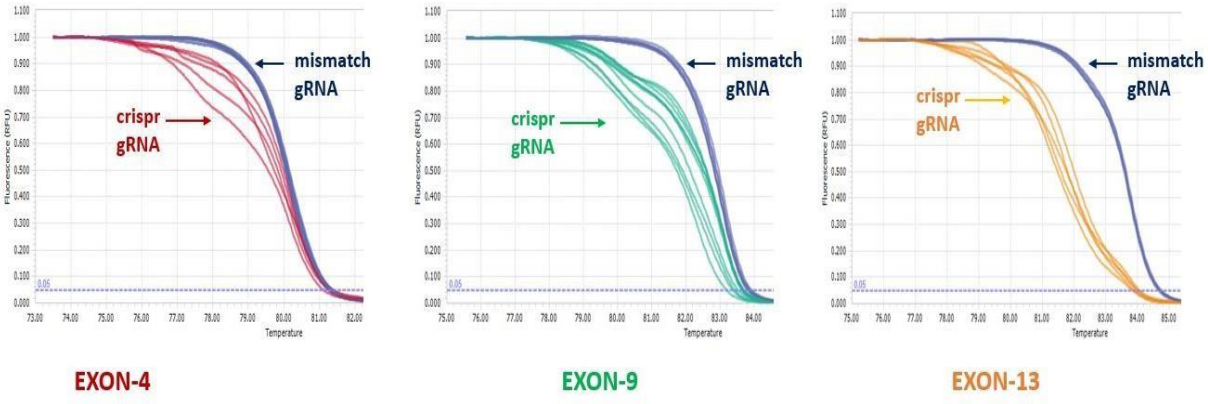

Figure 14: High-Resolution Melt Curve Analysis for *ints11* CRISPRant larvae and mismatch control guide RNAs

As shown in Figure 14, notably, all three CRISPR guide RNAs exhibited a shift in the melting curve, indicating successful mutagenesis at the desired loci. While the three-mismatch control guide RNAs did not show any shift. This serves as conclusive evidence that our methodology is reliable. Further, it is important to note that, for all the experiments performed, we confirmed the mutagenic efficacy of our injections through High-Resolution Melt (HRM) analysis.

5.2 *ints11* CRISPRant exhibits an increased level of unprocessed snRNAs

As the primary role of *INTS11* is snRNA processing, we aimed to assess the levels of unprocessed snRNA in our CRISPRant knockout model, we will further refer to these F0-CRISPRant larvae as *ints11*-KO for clarity purposes. We designed primers specifically to amplify the 3' unprocessed region of six zebrafish snRNAs (U1_4, U2, U2_1, U2_2, U5, and U12) and conducted RT-qPCR analyses using samples from 5-day-old whole larvae. The RT-qPCR results revealed a significant increase in the accumulation of unprocessed snRNA in the *ints11*-KO whole larvae compared to the mismatch controls (Figure 15A). To further investigate where this overexpression occurs, we examined the expression of unprocessed snRNAs in micro-dissected brains versus trunks (Figure 15B and 15C). Interestingly, we observed a robust increase in the expression of unprocessed snRNAs in *ints11*-KO in both the brain and trunks, indicating ubiquitous accumulation of unprocessed snRNAs. These results further validate our *ints11* zebrafish CRISPRant model, as *INTS11* disruption consistently leads to an increased accumulation of unprocessed snRNAs across various models, including *Drosophila*, *C. elegans*, and in vitro cell systems. (Ezzeddine et al., 2011; Gómez-Orte et al., 2019; Baillat et al., 2005; O'Reilly et al., 2014).

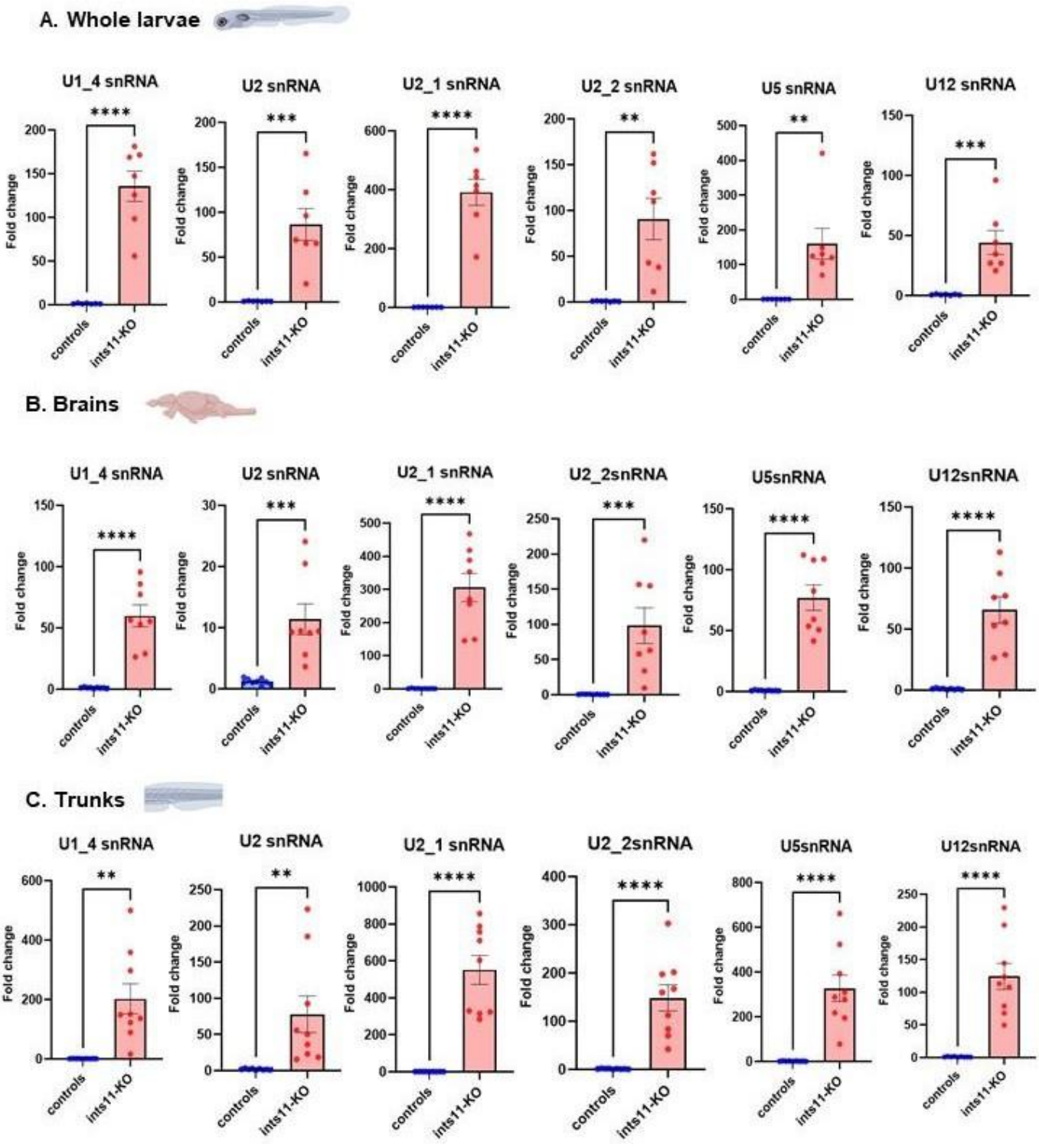

Figure 15: RT-qPCR analysis shows an increased accumulation of unprocessed snRNAs in *ints11* CRISPRant A) Whole larvae B) Brains C) Trunks

ddCt analysis, shown as fold-change to controls -T-test (n>35; N=3) for all samples

(* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001)

5.3 *ints11* CRISPRants die prematurely

After validating our *ints11* knock-out model by high-resolution melting assay and observing an increased accumulation of snRNAs, we performed a survival assay. Both *ints11*-KO and mismatch controls were raised in a 500ml beaker for 15 days at a temperature of 28ºC. Daily observations were made to track the number of deaths and to determine the genotype of the deceased larvae.

Notably, *ints11*-KO larvae showed a gradual increase in mortality starting from day **6.** Strikingly, by day 10, we observed a 50% mortality rate, and no larvae survived beyond day 14. In contrast, no deaths were recorded in the mismatch controls (Figure 16). These results suggest that the *ints11*-KO larvae die prematurely, although the exact cause of this lethality is not known. Interestingly, this premature death occurs at late larval stages, thus allowing us to perform neurodevelopmental observations during early embryonic/larval development.

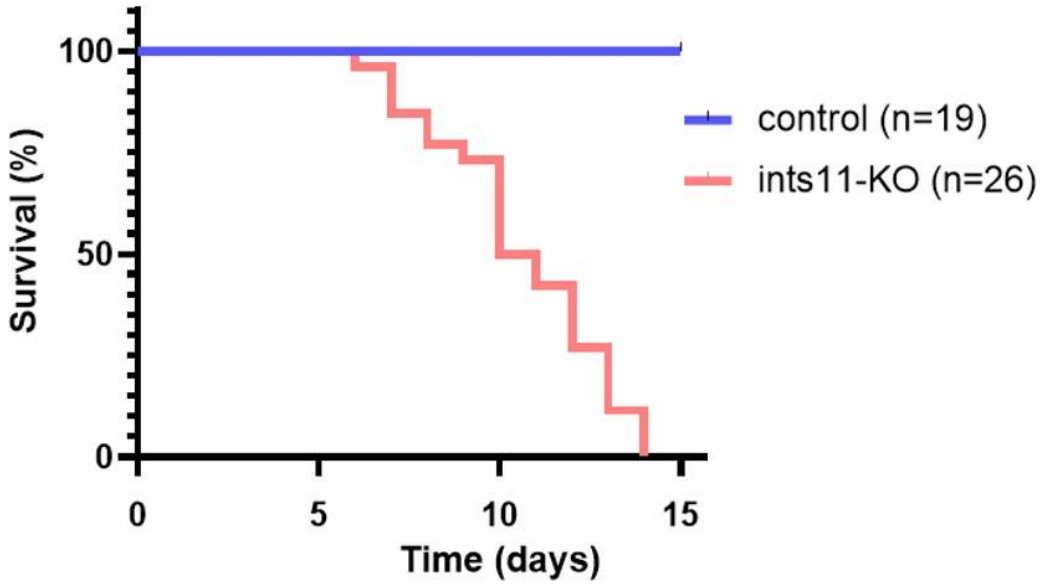

Figure 16: Survival Curve for *ints11* CRISPR-injected larvae over 14 days

5.4 *ints11* CRISPRant exhibits an impaired behavioural phenotype

Further, we performed a swim test using the DanioVision (Noldus Wageningen, The Netherlands). Larvae were habituated for 30 minutes before the start of the experiment, followed by a one-hour dark and one-hour light cycle. Interestingly, we noticed an impaired behavioural phenotype of *ints11*-KO larvae with dark-time hyperactivity and light-time hypoactivity (Figure 17 A and B). Importantly, this pattern of behavioural defects (hypoactivity under light, hyperactivity under dark) is reminiscent of the behaviour observed in other published zebrafish genetic models of neurodevelopmental encephalopathies (Samarut et al., 2018; Jamadagni et al., 2021; Butti et al., 2021). In addition, patients with *INTS11* mutations display hyperactivity and autism spectrum-like behaviours, providing further evidence that loss of *INTS11* function can result in impaired behavioural phenotypes.

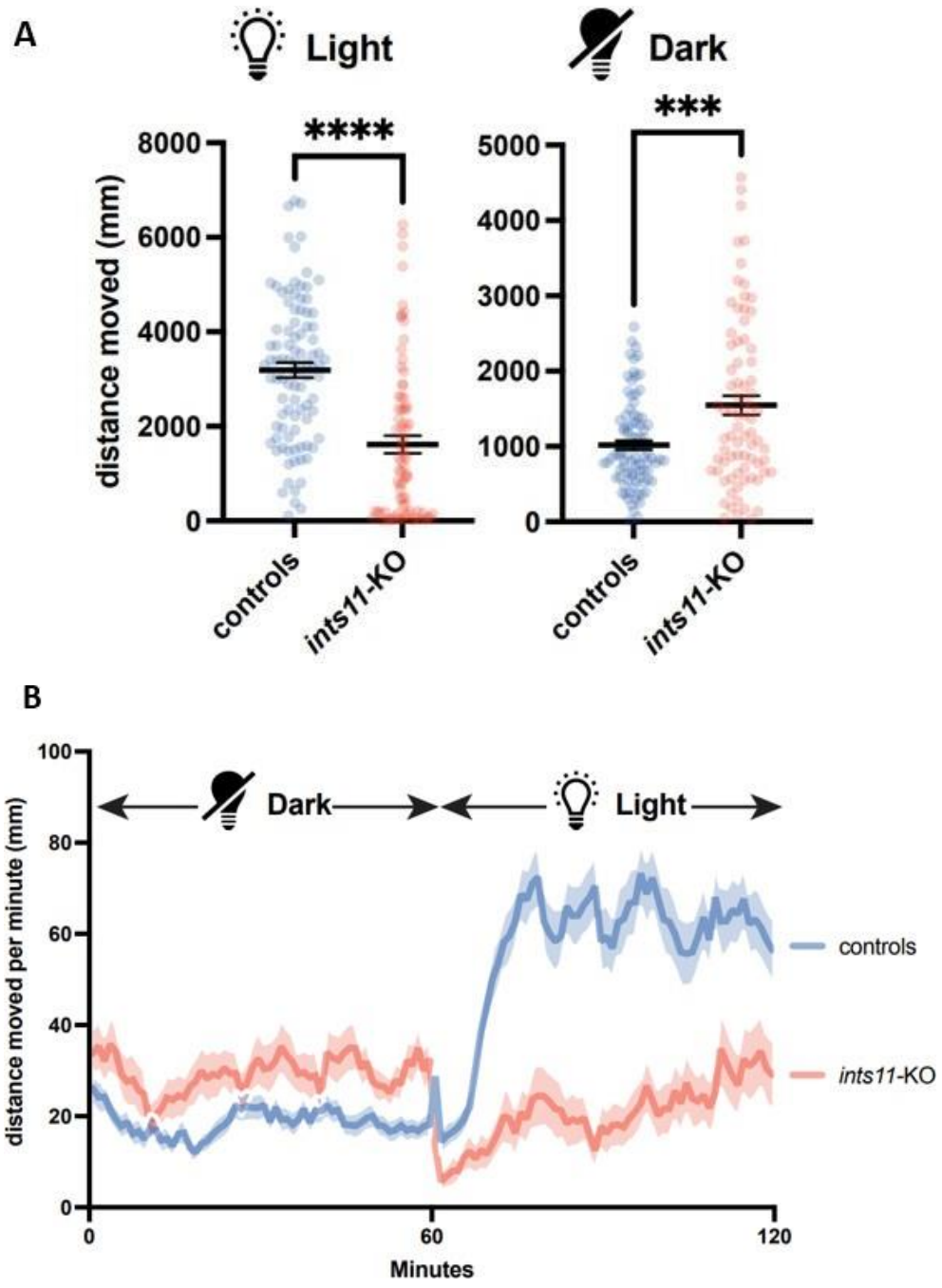

Figure 17: *ints11* CRISPRant larvae exhibit dark-time hyperactivity and light-time hypoactivity.

*A. Graph showing the distance moved (mm) per hour across dark (Student t test: ***P <0.0001) and light (Student t test: ****P <0.0001). Controls (n=93), ints11-KO (n=77)*

B. Measurements of the distance moved (mm) per minute period across one hour of dark and one hour of light. Controls (n=93), ints11-KO (n=77)

Moreover, we found a significant correlation between the mutagenic score of our *ints11* gRNAs and the level of hypoactivity observed in this phenotype, thus validating the specificity of this phenotype (Figure 18).

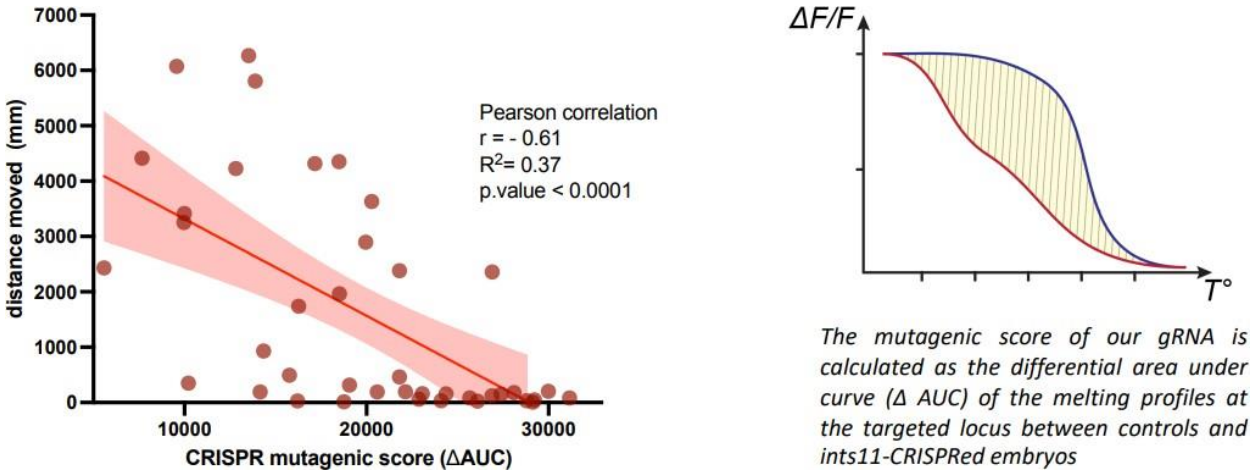

Figure 18: Pearson correlation graph showing the correlation of mutagenic score of the *ints11* guide RNAs and the distance moved in the light.

The CRISPR mutagenic score of the gRNA was calculated as the differential area under the curve of the (Δ AUC) of the high-resolution melting profiles at the targeted locus between controls and the ints11- CRISPRed larvae.

5.5 *ints11* CRISPRant have smaller eye size

Furthermore, we performed a general phenotypic assessment, closely monitoring *ints11*-KO and mismatch control larvae daily for any apparent morphologic abnormalities. However, as shown in Figure 19 A, *ints11*-KO larvae showed no noticeable morphologic differences at 5 dpf, as suggested by an average body and head size (Figure 19 B).

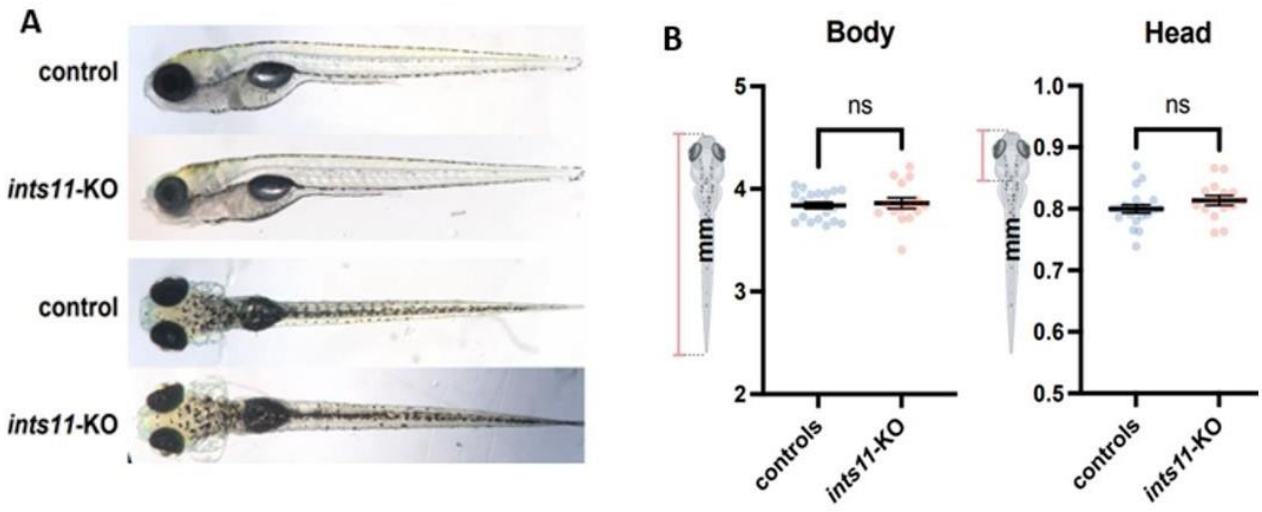

Figure 19: *ints11* CRISPRant have smaller eye size

A. Morphologic phenotyping of ints11 knockout animals.

B. Quantification of body size and head length
Student t-test, Controls (n=20), ints11-KO (n=16)

Given that patients with mutations in the *INTS11* gene often manifest clinical symptoms such as optic atrophy and visual impairment, we examined eye development in our zebrafish mutants by measuring eye diameter and inter-eye distance. Notably, we found that eye diameter was significantly reduced in *ints11*-KO larvae compared to controls, suggesting a specific impact of *ints11*-KO on the central nervous system. However, the inter-eye space distance remained normal (Figure 20 A and B).

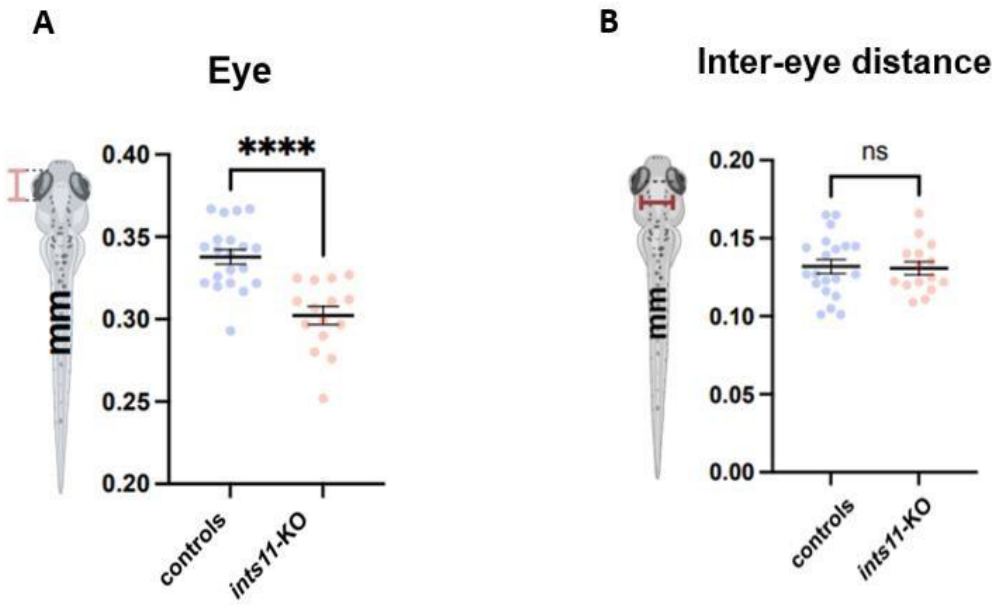

Figure 20: Quantification of eye diameter and Inter-eye distance in *ints11*-KO

A. *Quantification of Eye Diameter. Student t-test: ****P < 0.0001. Control (n=20), ints11-KO (n=15)*
B. *Quantification of Inter-Eye Distance. Student t-test. Controls (n=20), ints11-KO (n=15)*

5.6 *ints11*-CRISPRant larvae have smaller brain

As the next crucial step, we aimed to investigate the impact of *ints11* loss of function at the central nervous system level, particularly brain development. We estimated the brain size of *ints11*-KO larvae using the transgenic *Tg[elavl3:GFP]* line, which expresses green fluorescent protein (GFP) in post-mitotic neurons (Khuansuwan et al., 2019). In *ints11*-KO larvae, we noted a significant reduction in overall brain size compared to the mismatch control group (Figure 21 A and B).

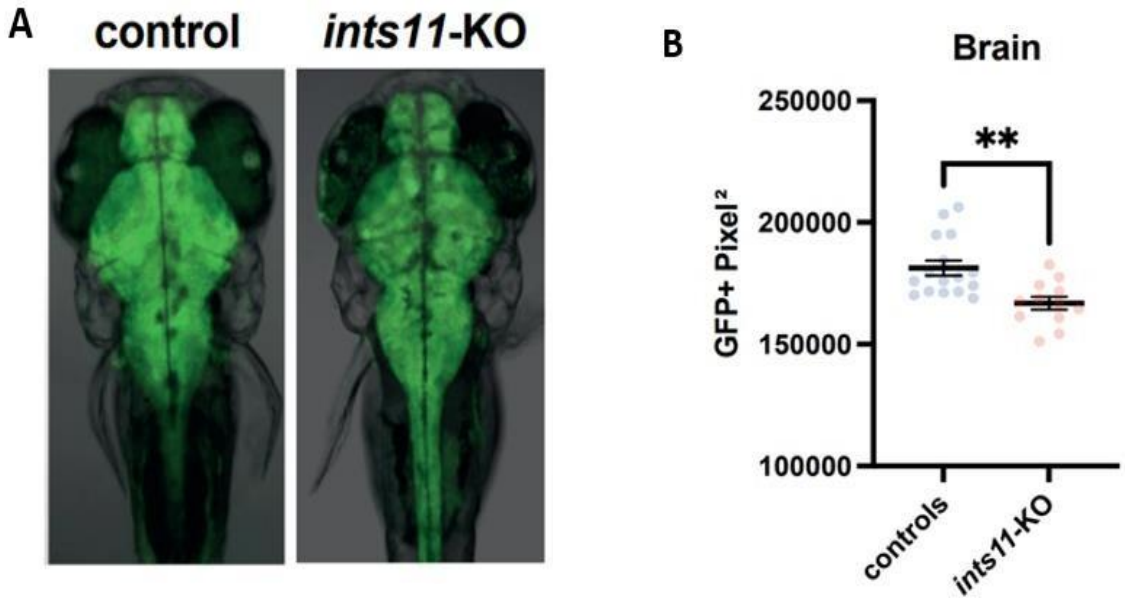

Figure 21: *ints11*-CRISPRant larvae display smaller brain

A. 5 dpf Larval Brain Images from elavl3: GFP

*B. Quantification of brain size (student t-test - ** p<0.01) Controls (n=18), ints11-KO (n=13)*

Further, we measured individual larval brain regions, namely the forebrain, midbrain, hindbrain and brain stem. Interestingly, we noticed a reduced midbrain size in our *ints-11* KO compared to the controls (Figure 22).

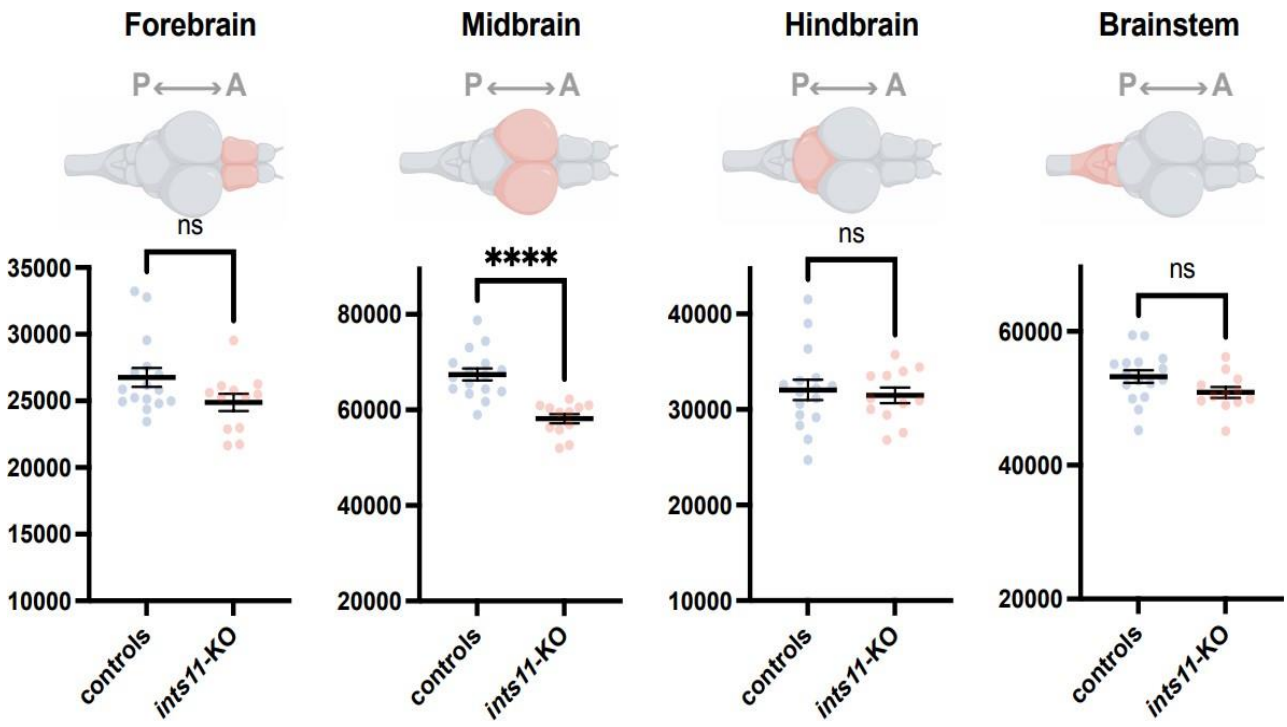

Figure 22: Quantification of different brain regions

*The forebrain, hindbrain and brainstem show a trend toward a decreased size, albeit not significant. The midbrain size was reduced in our ints-11 KO (Student t-test: ****P <0.0001) Controls (n=18) and ints11-KO (n=13).*

Notably, *INTS11* loss of function patients exhibit distinct alterations in brain structure, which include atrophy in the cerebellar and basal ganglia regions and progressive cerebellar hypoplasia. Additionally, they manifest other notable features, such as leukoencephalopathy (a condition involving damage to the brain's white matter), dilated ventricles, and a thin corpus callosum. While the clinical phenotype does not explicitly mention changes in the midbrain, it is important to consider that the effect on the midbrain may not be particularly specific in zebrafish. Instead, the phenotype appears more noticeable in the midbrain than other brain regions, as it is a relatively larger part of the brain in 5-day-old zebrafish larvae. Moreover, it is crucial to recognize that alterations in

neighbouring brain regions, such as the cerebellum and basal ganglia, can have far-reaching consequences throughout the entire brain.

5.7 *ints11*-CRISPRant larvae have reduced neuronal content

Further, we aimed to explore deeper into the midbrain, specifically focusing on the optic tectum, which is the more prominent structure of the zebrafish larval brain. Notably, we observed a reduced number of neuronal cell bodies in the optic tectum of *ints11*-KO larvae compared to controls (Figure 22A).

When we quantified these neuronal cell bodies, we observed a significant reduction in their count in *ints11*-KO larvae compared with the control group. (Figure 23 B). This finding strongly correlates with a severe neurodevelopmental problem (Hall and Tropepe, 2018).

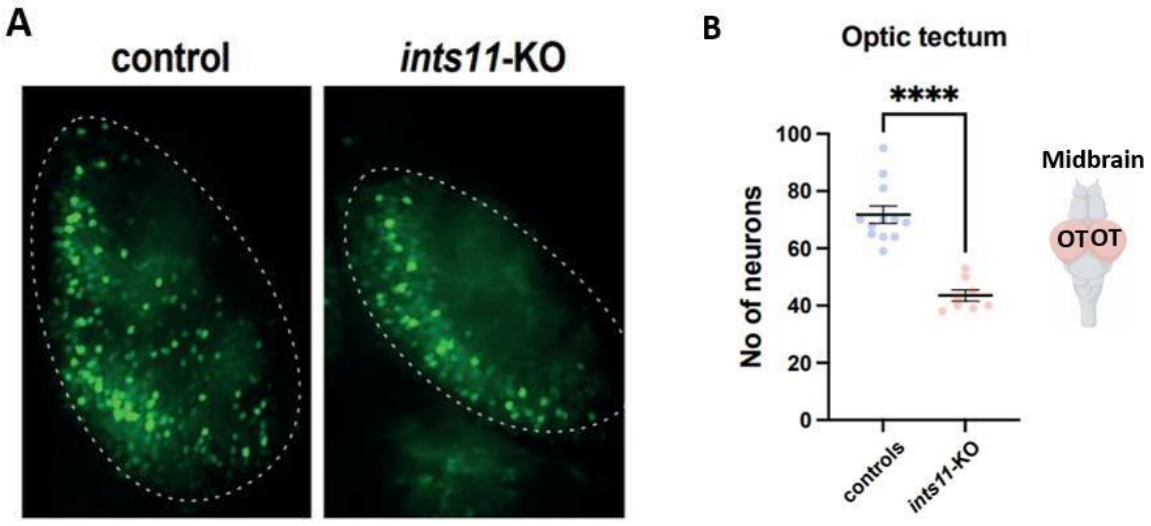

Figure 23: *ints11*-CRISPRant larvae exhibit reduced neuronal content

A. Confocal imaging of 5dpf larvae from elavl3: GFP transgenic (at a 20x zoom)

B. Quantification of the optic tectum
*(Student t-test: ****P <0.0001) Controls (n=12), ints11-KO (n=8)*

5.8 *ints11*-CRISPRant larvae have a reduction in the size of the cerebellum

Patients with *INTS11* loss of function were reported with ataxic problems and exhibited smaller cerebellums. To investigate this further, we checked the integrity of the cerebellum in our zebrafish model by conducting immunostaining against anti-parvalbumin-7 (PAV7), a marker for Purkinje cells crucial for cerebellar function. Moreover, ataxic patients are frequently associated with a smaller cerebellum, and in many cases of ataxia, Purkinje cells in the cerebellum can be affected (Xia et al., 2013; Hoxha et al., 2018).

When we quantified the size of the cerebellum by PAV7+ positive staining, we found a smaller cerebellum size of *ints11*-KO larvae compared to the mismatch controls, as shown in Figures 24 A and B. This observed reduction in cerebellar size in our zebrafish model closely mirrors the smaller cerebellums often observed in patients, further strengthening the link between our *INTS11* loss-of-function in vivo and the clinical phenotype of patients.

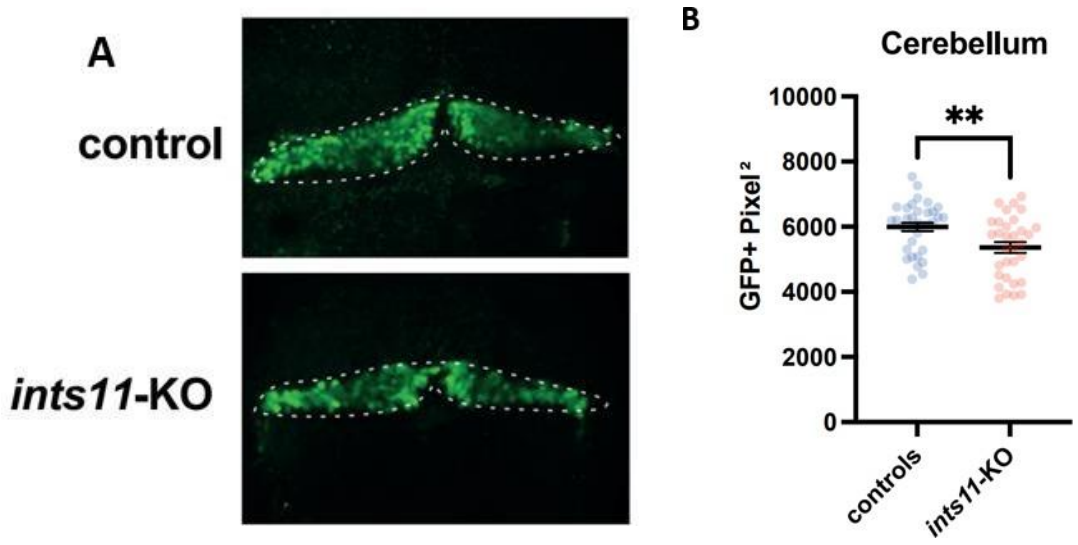

Figure 24: *ints11*-CRISPRant larvae have a reduction in the size of the cerebellum

A. Confocal imaging of 5dpf larvae showing reduced cerebellum size of the ints11 CRISPRant (bottom) compared to the mismatch control (top)

Immunostaining against Purkinje cells, PAV7, is shown in green.

B. Quantification of the cerebellum

*(Student t-test: **P <0.0040) Controls (n=33), ints11-KO (n=34)*

Overall, *ints11* loss-of-function causes a severe neurodevelopmental phenotype in zebrafish, characterized by a reduced brain size and a reduced neuronal content particularly at the level of the cerebellum, associated with behavioural abnormalities. Altogether, these data support the role of *INTS11* in brain development and are consistent with neurodevelopment delays described in patients carrying deleterious mutations in this gene.

CHAPTER 3: CONCLUSION

One of the major goals of precision medicine is to enhance patient diagnosis and customize treatments based on individual genetic profiles. However, the diagnosis becomes more challenging if the underlying genetic cause remains unknown. Moreover, a plethora of genetic variants have been reported with the advent of next-generation sequencing. Classifying these variants as pathogenic or benign remains a persistent challenge. Most of these variants fall under the "Variants of Unknown Significance" category. Functional characterization plays a crucial role in unravelling the implications of genetic variation, especially in cases of uncertain clinical significance or unknown genetic origins. This characterization helps researchers to determine whether a genetic variant is associated with a specific disease.

Our primary focus was on the *INTS11* gene, which serves as the catalytic subunit of the Integrator complex. Recently, we collaborated with a geneticist who had identified multiple patients with *INTS11* mutations. These patients exhibit a wide range of symptoms, including global developmental delay, autistic behaviours, hyperactivity, ataxia, optic atrophy, visual impairment, atrophy in various regions of the brain, smaller cerebellum, cerebellar hypoplasia, and delayed myelination.

Despite these clinical observations, there is a lack of functional evidence to confirm the pathogenicity of mutations in this gene. When we began this project, there were no reported cases of *INTS11* mutation in humans. However, it is important to note that, in the final stages of the project, a study found that *INTS11* mutations were linked to neurodevelopmental issues (Tepe et al., 2023). However, the functional characterization of

INTS11 loss-of-function in this study is limited. It lacks validation at the level of snRNA processing and assessing the effects at the central nervous system level *in vivo.*

In this study, we generated the first vertebrate model of *INTS11* in zebrafish using CRISPR/CAS9 technology. Specifically, we developed *ints11* F0 CRISPRants using three guide RNAs targeting exon 4, exon 9, and exon 13, which encode essential protein functional domains. As controls, we used embryos injected with the same mutagenic molecular cocktail but with 5-mismatch control, gRNAs, to exclude any potential variability that may arise from injections. We confirmed the efficacy of all the guide RNAs using high-resolution melting assay (HRM) genotyping.

Our zebrafish F0 CRISPRant model displayed several characteristics resembling the clinical phenotypes observed in patients. First, we proved that *INTS11* function was disrupted in our model by showing an increased accumulation of unprocessed snRNAs in our *ints11*-CRISPRants, consistent with previous findings in other models, such as *Drosophila*, *C. elegans*, and in vitro cell systems (Baillat et al., 2005; O'Reilly et al., 2014; Ezzeddine et al., 2011; Gómez-Orte et al., 2019). Our results from the survival assay showed that all of the *ints-11* CRISPRants die before reaching day 14, which is a significant sign that they are not healthy. Moreover, a similar pattern of larval stage death was observed in the *ints11 Drosophila* model, published during the project's final phase (Tepe et al., 2023). These results suggest that the *INTS11* gene may be crucial in maintaining proper development.

Furthermore, our study revealed behavioural abnormalities in *ints11*-CRISPRants, characterized by hypoactivity under light conditions and hyperactivity in the dark. These behavioural patterns closely resemble those observed in previously reported zebrafish

genetic models associated with neurodevelopmental encephalopathies (Samarut et al., 2018; Jamadagni et al., 2021; Butti et al., 2021). These similarities in behavioural phenotypes provide valuable support for our findings and suggest that disruption of the *INTS11* gene in our zebrafish model may lead to neurodevelopmental anomalies.

Most patients with *INTS11* mutations had developmental problems; therefore, we closely observed our *ints11*-CRISPRants for morphological changes. While no apparent phenotypic abnormalities regarding body and head size were observed, we found that eye diameter was significantly reduced in *ints11*-KO larvae compared to controls. This reduction in eye size suggests that the knockout of the *ints11* gene may have a specific impact on the central nervous system. Interestingly, *ints11*-KO also exhibited reduced brain area associated with reduced neuronal content in the optic tectum. Finally, immunostaining for Purkinje cells revealed a significant decrease in the size of the cerebellum, similar to that observed in the patients.

Altogether, these findings provide evidence that the disruption of *INTS11* function leads to neurodevelopmental abnormalities. Our study is a prominent example of functional genetic characterization in vivo, demonstrating how simple animal models, such as zebrafish, can validate genetic variants' pathogenicity. In particular, the results from our study could be used for the accurate diagnosis and treatment of patients with mutations in the *INTS11* gene. This could significantly reduce healthcare costs by streamlining diagnostics and preventing misdiagnosis.

Note: We are currently submitting our results to publication.

CHAPTER 4: DISCUSSION

1.1 Unsolved questions in the Integrator complex

Our study provides functional evidence supporting the role of the *INTS11* gene in neurodevelopmental problems. However, there are numerous unresolved questions regarding the Integrator complex. The Integrator complex is a multi-subunit protein, and although some subunits have been studied more extensively than others, the exact function of each subunit remains an ongoing area of research (Sabath et al., 2020). The *INTS11* gene cleaves various noncoding RNAs, like snRNAs, enhancer RNAs and nascent mRNAs produced by protein-coding genes. However, how the Integrator complex is recruited to each snRNA locus remains unknown but only to a specific subset of protein-coding genes and enhancers (Mendoza-Figueroa et al., 2020).

Further research is needed to elucidate whether Integrator activity at protein-coding loci and enhancers is always dependent on *INTS11* RNA endonuclease or whether other subunits play a role in RNA cleavage activity (Mendoza-Figueroa et al., 2020). Moreover, exploring the role of *INTS9* is of particular interest, as it may provide insights into whether disruption of non-catalytic subunits can also lead to developmental defects.

1.2 Significance of *INTS11* gene interactions

Recent studies have shed light on the interactions between *INTS11* and other genes in the context of neurodevelopmental disorders. Notably, *INTS11* has emerged as a novel partner of the *WDR73* gene, associated with Galloway–Mowat syndrome, a complex phenotype characterized by neurological symptoms and renal abnormalities (Tilley et al., 2021). Moreover, *INTS11* has been found to interact with *BRAT1*, a gene associated with

neurodevelopmental and neurodegenerative disorders characterized by diverse clinical presentations (Cihlarova et al., 2022).

Interestingly, individuals carrying variants of both *BRAT1* and *INTS11* share common symptoms such as cerebellar atrophy, ataxia, and cognitive impairment. This suggests that certain *INTS11* variants may disrupt this interaction, potentially leading to these clinical features. However, the exact molecular mechanisms by which these *INTS11* variants lead to this phenomenon remain a subject of ongoing research. Furthermore, these studies emphasize the importance of identifying genes interacting with *INTS11*. We are currently collaborating with Dr. Hana Hanzlikova, a researcher at the Czech Academy of Sciences, to better understand the pathogenic role of *INTS11* and *BRAT1* mutations in the context of this disease. Investigating these interactions provides valuable insights into the molecular mechanisms of related diseases, helping us better understand and diagnose them.

1.3 Possible mechanisms leading to developmental abnormalities

The precise mechanisms by which mutations in the Integrator complex give rise to developmental abnormalities remain a subject of ongoing research. However, a possible hypothesis is that as the Integrator complex plays a crucial role in the processing of small nuclear RNAs (snRNAs), alterations in the processing of specific snRNA transcripts may have the potential to contribute to developmental defects (Mendoza-Figueroa et al., 2020).

Moreover, recent studies have highlighted the role of the Integrator complex in regulating protein-coding transcription to maintain normal development. When the Integrator complex is either depleted or mutated, it can lead to the misregulation of hundreds to thousands of messenger RNAs (mRNAs) (Stadelmayer et al., 2014; Tatomer

et al., 2019). For example, in a human fibroblast model, mutations in the Integrator complex led to over 3000 mRNA expressions, with 215 genes showing alterations in alternative splicing patterns (Oegema et al., 2017). This underscores the significance of the Integrator complex in controlling gene activity during development and highlights how mutations in this complex can significantly affect normal development.

Overall, our *ints11* zebrafish CRISPRant model has already provided valuable insights into the role of *INTS11* in neurodevelopmental disorders. However, further research is essential to unravel the precise molecular mechanisms through which *INTS11* disruption leads to neurodevelopmental phenotypes in order to develop targeted therapies.

Interestingly, although *INTS11* is expressed ubiquitously in humans, it is specifically associated with neurodevelopmental problems. It may be possible that, despite its ubiquitous expression, *INTS11* may have tissue-specific functions, especially in the context of neurodevelopmental problems. Mutations in this gene can significantly impact brain tissues or neuronal cells, potentially leading to neurological symptoms.

To expand our understanding using our zebrafish *ints11* CRISPRant model, it will be interesting to visualize the spatial distribution of *ints11* mRNA during various developmental stages of zebrafish using in situ hybridization. This will shed light on the expression patterns of *INTS11* during zebrafish development.

Further studies are needed to investigate *INTS11* expression in various zebrafish tissues, including brain and non-neuronal tissues, using in situ hybridization and quantitative PCR (qPCR). This will enable us to determine whether *INTS11* is highly expressed in brain tissues and neurons.

Additionally, we can also explore *INTS11* expression in various brain cell types, including glial cells (astrocytes, oligodendrocytes, and microglia) and neural progenitor cells. These cell types play crucial roles in neurodevelopment, and studying *INTS11* expression across various brain regions may reveal its distinct roles in neurodevelopmental processes.

It is also essential to study how *INTS11* affects the processing of snRNAs and mRNAs in zebrafish and to explore its interactions with other genes in the context of neurodevelopment. Our qPCR results showed increased expression of unprocessed snRNAs in *ints11*-KO compared to the controls, both in the brain and trunks, indicating a ubiquitous accumulation of unprocessed snRNAs.

Further studies are needed to determine whether increased levels of unprocessed snRNAs are specific to certain types of brain cells, such as Purkinje cells, cerebellar granule cells, and glial cells. Immunofluorescence and in situ hybridization techniques can be used to investigate the accumulation of unprocessed snRNA in particular brain cells in *ints11*-KO and controls. This sheds light on the role of *INTS11* in snRNA processing and its potential impact on neurodevelopment.

To further investigate the molecular pathways involved in snRNA processing and the impact of *INTS11* depletion, proteomic analysis using mass spectrometry should be performed to explore the proteins and factors associated with snRNA biogenesis, modification, and processing (Wein et al., 2020). This will help us to understand whether *INTS11* depletion disrupts the interactions and functions of these critical components.

Moreover, by investigating alternative splicing events through techniques such as RNA sequencing (RNA-Seq), we can potentially identify specific pathways affected by *INTS11* mutations, providing insights into how *INTS11* affects mRNA processing and contributes to neurodevelopmental disorders (Hostelley et al., 2017).

1.4 Challenges in validating point mutations in zebrafish

In this study, we demonstrated the potential of zebrafish as a model for loss-of-function mutations. However, one major limitation of our study is that we only modelled a general loss of function of *INTS11* and not missense mutations specifically, although many patients carry these point mutations. Studying the effects of missense mutations in zebrafish is technically challenging compared to generating knockout models. One significant limitation is that the protein residues targeted by the mutation may not be found in zebrafish. Additionally, if an ortholog gene is present, a specific missense mutation might not have the same impact in zebrafish as in humans (Carrington et al., 2022).

One approach to studying missense mutations involves injecting the mRNA of a patient's variant into a knockout genetic background. If a wild-type variant can rescue a particular phenotype, we can assess whether the pathogenicity of a specific variant is linked to the absence of phenotypic rescue.

For example, in a recent study involving the *IRF6* gene, which is associated with orofacial cleft syndromes, a rescue assay was performed using a knockout model of *irf6* in zebrafish. They injected mRNA from more than 30 human *IRF6* missense variants and confirmed the pathogenicity of variants classified as “pathogenic” using computational tools. However, there were discrepancies in interpretation for about 50% of the variant

classified as “likely pathogenic” (Li et al., 2017). This method is transient and does not always perform well. Nevertheless, it remains a valuable tool many researchers use to determine whether a patient's mutation can rescue the knockout phenotype, thereby inferring a pathogenic score for the mutation.

Further, an alternative approach is possible, such as recombining the entire human coding DNA sequence as a transgene at the endogenous locus. However, this genetic engineering process would be time-consuming and lack specificity as the human transgene would be inserted randomly in the zebrafish’s genome.

Interestingly, recent studies have successfully developed a humanized version of nematode worms, creating a valuable *in vivo* platform for assessing the pathogenicity of Variants of Unknown Significance (VUS) in the *STXBP1* gene associated with epileptic syndromes. Researchers have used the CRISPR-Cas9 technique to introduce mutations in *Caenorhabditis elegans* to investigate the pathogenicity of variants (Zhu et al., 2020).

Another study employed a humanized *C.elegans* model to characterize specific Variants of Unknown Significance (VUS) in the *KLC4* gene linked to Hereditary Spastic Paraplegia, a group of degenerative neurological disorders. These studies emphasize the potential of *in vivo* assays for rapid and precise assessment of VUS as either pathogenic or benign (Gümüşderelioğlu et al., 2023).

1.5 Our dream of generating patient-specific genetic avatars for tailoring medication

Considering the challenges and limitations of using zebrafish as a model for point mutations, we explored an innovative approach to create patient-personalized genetic models in zebrafish. As a side project, we aimed to establish a proof-of-concept study to

generate patient-specific genetic avatars for epilepsy, tailor anti-epileptic medication, and advance precision medicine (Figure 25).

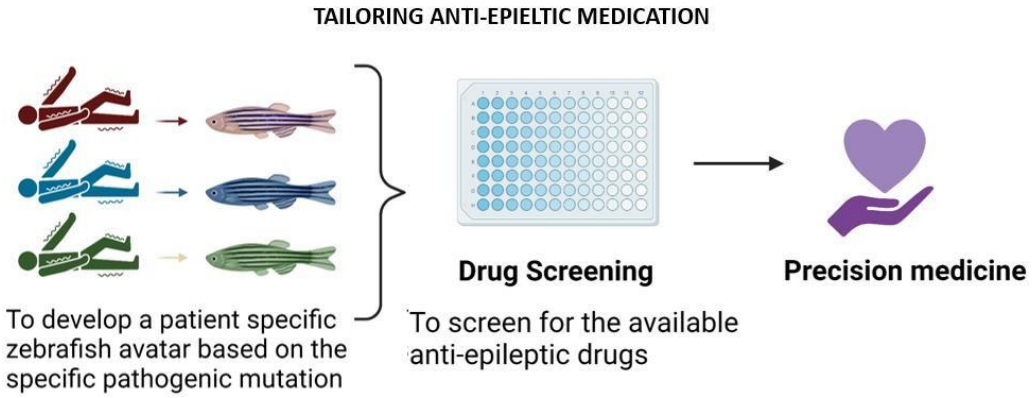

Figure 25: Overview of tailoring medication with zebrafish genetic avatars

We were particularly interested in employing *in vivo* recombinase-mediated cassette exchange (RMCE) in zebrafish. RMCE is a process that allows the swapping of large genomic regions, provided they are flanked by an identical pair of recombinase sites in the presence of a recombinase enzyme (Turan et al., 2013).

Of particular interest, we chose to work with the *gabra1* gene, a well-established epilepsy-causing gene known for its complex genotype-phenotype spectrum. Our laboratory has previously developed a *gabra1* knockout zebrafish model and extensively characterized its epileptic phenotype.

Initially, we used the Cre-loxP recombination system to achieve recombinase-mediated cassette exchange. This technique uses Cre recombinase enzyme to target specific DNA sequences (loxP sites) and exchange genetic material between these sites (McLellan et al., 2017). We employed the CRISPR-cas9 knock-in technique to insert a specific lox site into the endogenous zebrafish *gabra1* gene. Subsequently, we attempted

recombination by introducing a second lox site containing a fluorescent reporter. Our preliminary experiments used red fluorescent protein (RFP) instead of patient cDNA. Unfortunately, we could not achieve a successful recombination with our fluorescent reporter using this method. The results of our proof-of-concept study of Cre-loxP recombination are available in Appendix A.

Despite our initial challenges, we are now exploring the same strategy using serine-recombinases, particularly phic31 recombination, known for its efficiency in driving site-specific recombination events (Bateman et al., 2006). We believe that this approach holds great promise for achieving our goal of generating patient-specific genetic avatars in zebrafish.

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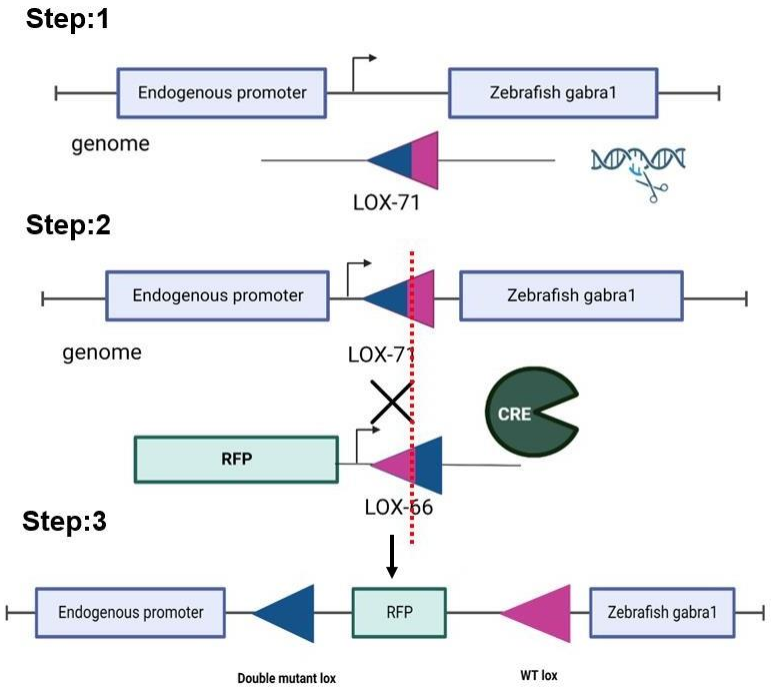
APPENDIX -A

Generating patient-specific genetic avatars: A proof of concept using the cre-lox recombination process

In the cre-lox transgenic approach, to achieve irreversible recombination, we inserted mutated versions of the lox site, specifically the lox71 sequence, at the endogenous zebrafish *gabra1* locus using CRISPR-cas9 knock-in. The lox71sequence is a half-mutated loxP site that can recombine with another half-mutated site, lox66, resulting in the formation of one loxP site at the 3'end and a mutated loxP at the 5'end. This recombination event avoids excision and enables stable integration.

Once we generated a stable transgenic zebrafish line carrying a Lox71 site Tg[gabra1_Lox71], we tried to recombine the lox 71 site with a second lox site, namely lox66, encompassing the red fluorescent protein (RFP) reporter gene (Figure A).



Step 1 *Insertion of lox71 site at the 5'UTR of the zebrafish gabra1 locus by CRISPR-CAS9*

Step 2 *Recombination of the lox71 site with lox66 encompassing RFP (florescent reporter)*

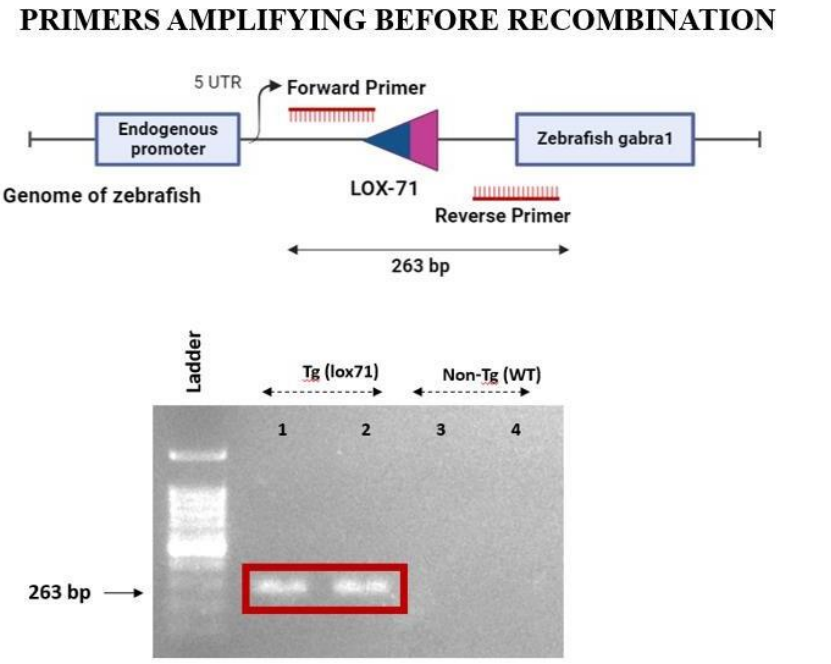
Step 3 *Successful recombination should result in the visible expression of red fluorescent protein (RFP) and yield a double mutant lox site (which cannot recombine anymore) along with a regular lox site*

lox71 sequence: taccgTTCGTATAGCATACTTTATACGAAGTTAT

lox66 sequence: ATAACTTCGTATAGCATACTTTATACGAAcggta

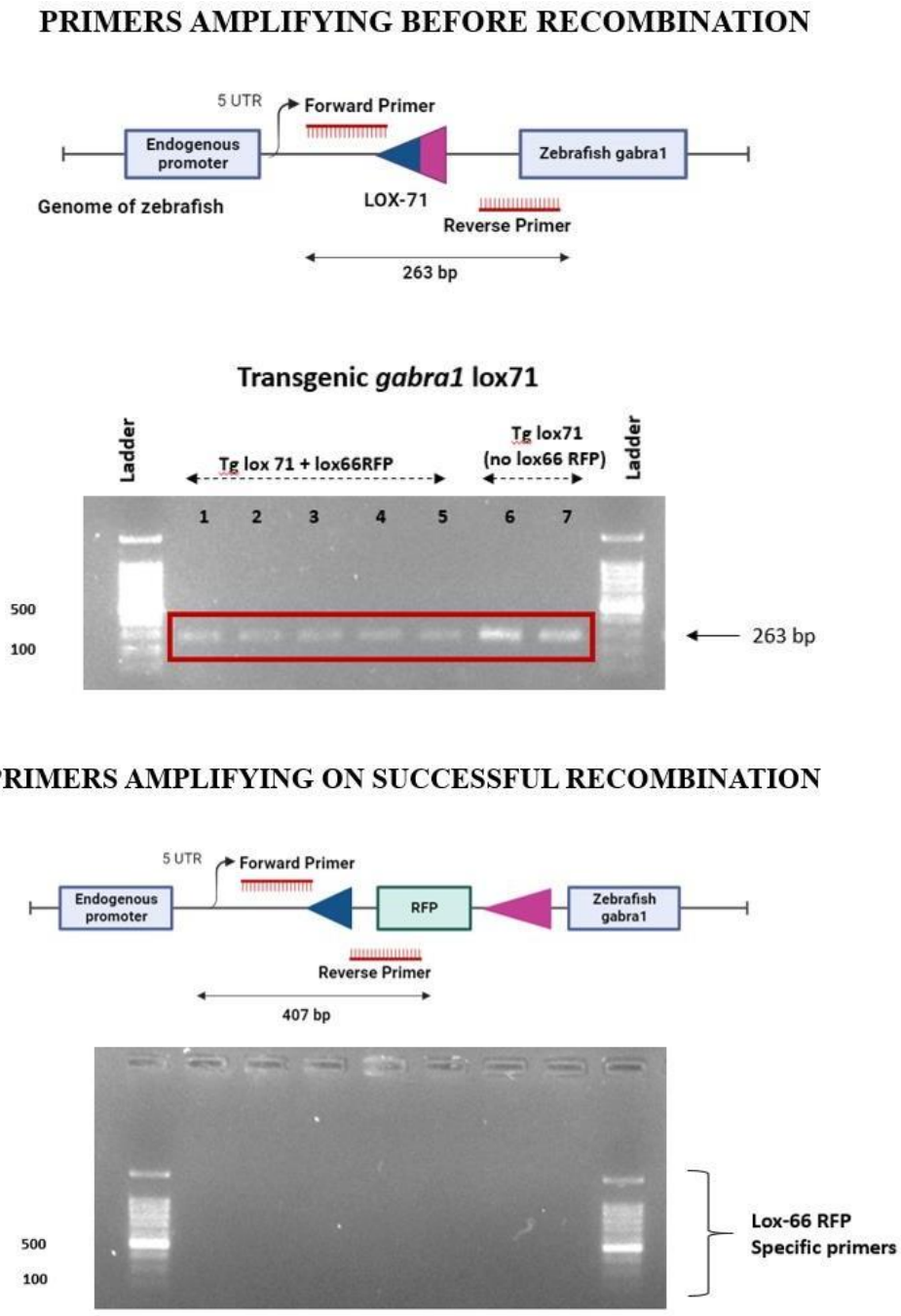
Figure A: Outline of the lox71-lox66 recombination process

As we could not visually detect red fluorescent protein under a confocal microscope, we validated the recombination process by using specific PCR primers designed to detect the RFP.



Agarose gel image showing band specificity comparing transgenic vs non-transgenic embryos

Lanes 1-2 shows transgenic positive controls from lox 71 transgenic fish
Lanes 3-4 shows wildtype controls that have no bands with the primers specific to the transgene

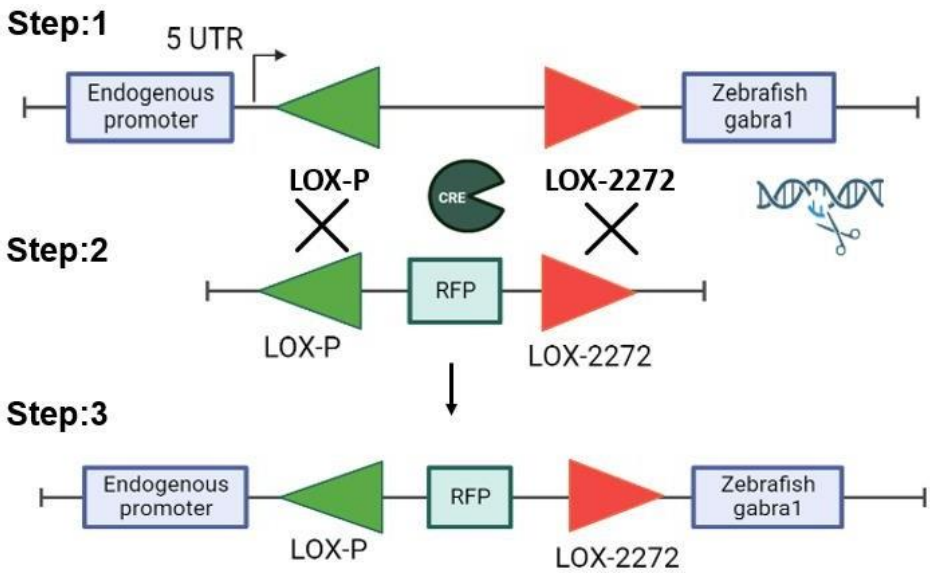


Agarose gel image of embryos containing *gabra1_Lox71* injected with lox66-RFP

TOP: Primers specific to the lox -71 transgenic sequence
Lanes 1-5 shows bands specific to the transgenic lox-71 injected with lox66 RFP
Lanes 6-7 shows bands specific to the transgenic lox-71 but uninjected with lox66 RFP
BOTTOM: Indicates samples ran with specific primers to check if the recombination with lox66RFP was successful. The expected band size is around 407bp. But no bands were observed.

Figure B: Agarose gel images of the lox71-lox66 strategy

As a second strategy, we also tried inserting two distinct lox sites, loxP and lox2272, at the zebrafish *gabra1* locus using CRISPR-cas9 knock-in. Then, we tried recombining with a second construct consisting of loxP_RFP_lox2272 (Figure C).



Step 1 *Insertion of a LOXP and LOX2272 site by CRISPR-CAS9 (Knock-in) at the zebrafish gabra1 locus*

Step 2 *Recombination of LOXP-LOX2272 site with a donor cassette containing LOXP-LOX2272 with RFP (fluorescent reporter)*

Step 3 *Successful recombination should result in the visible expression of red fluorescent protein (RFP)*

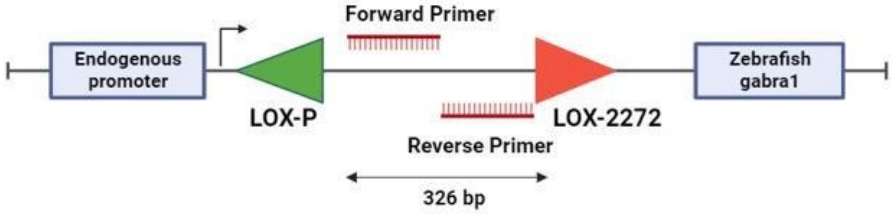
LoxP sequence: ATAACTTCGTATAATGTATGCTATACGAAGTTAT

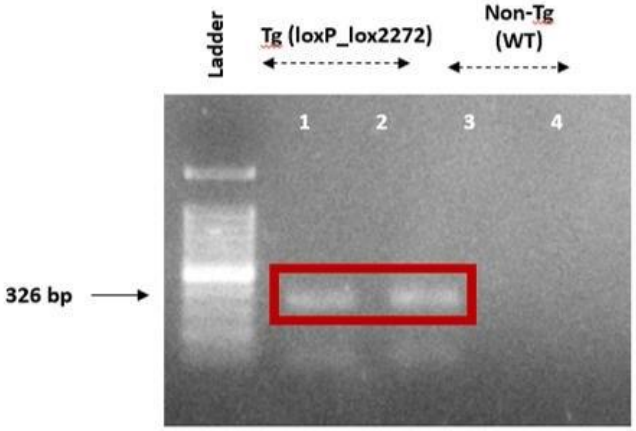
Lox2272 sequence: ATAACTTCGTATAAAGTATCCTATACGAAGTTAT

Figure C: Outline of the loxP-lox2272 recombination process

Similar to the previous approach, we could not detect red fluorescent protein using a confocal microscope, we validated the recombination process by using specific PCR primers designed to detect the RFP.

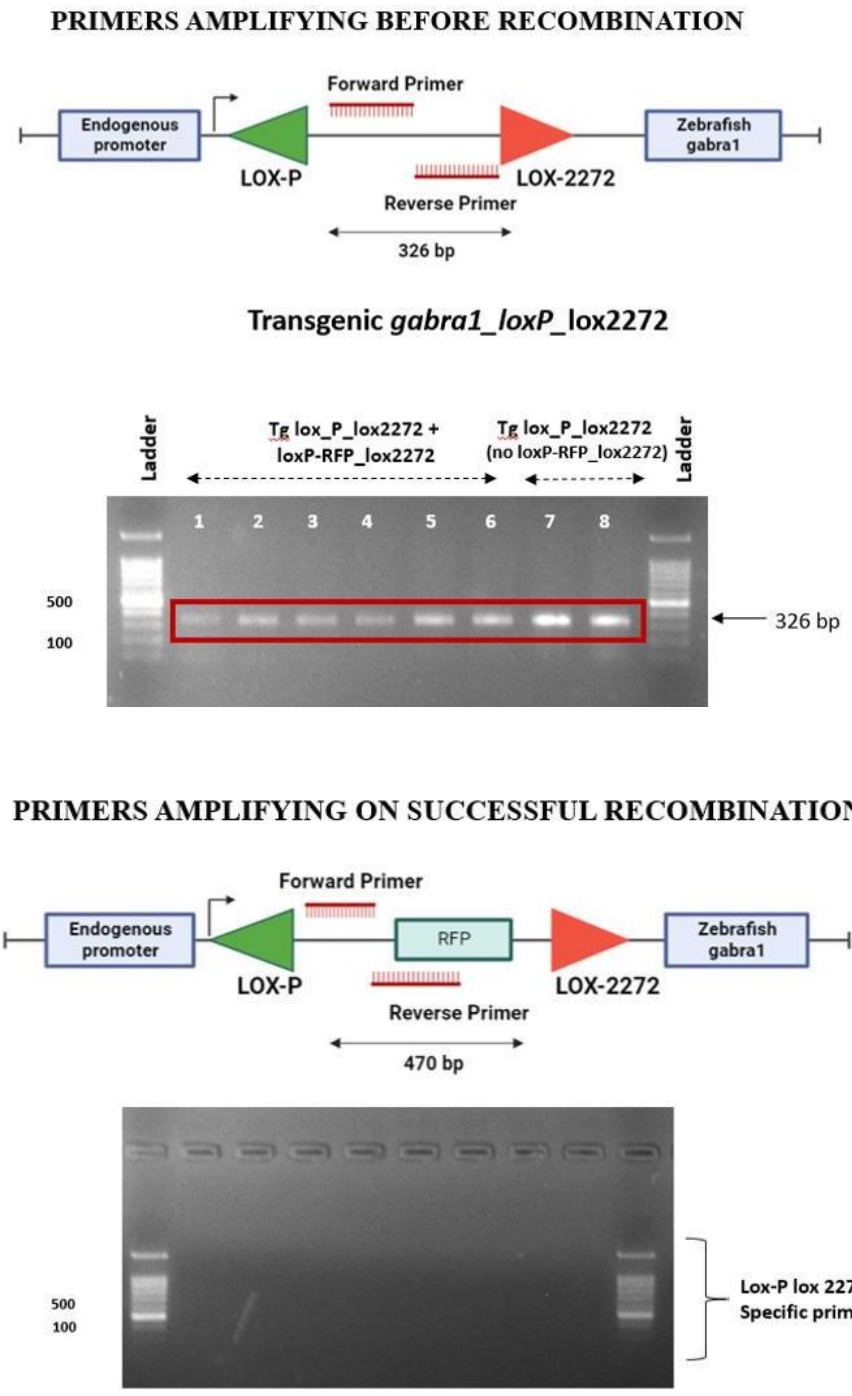
PRIMERS AMPLIFYING BEFORE RECOMBINATION





Agarose gel image showing band specificity comparing transgenic vs non-transgenic embryos

Lanes 1-2 shows transgenic positive controls from loxP_lox 2272 transgenic fish
Lanes 3-4 shows wildtype controls that have no bands with the primers specific to the transgene



Agarose gel image of embryos containing *gabra1*_loxP_*lox2272* injected with loxP-RFP_lox2272

TOP: Primers specific to the loxP_lox2272 transgenic sequence
Lanes 1-6 shows bands specific to the transgenic loxP_lox2272 injected with loxP-RFP_lox2272
Lanes 7-8 shows bands specific to the transgenic loxP_lox2272 but uninjected with loxP-RFP_lox2272
BOTTOM: Indicates samples ran with specific primers to check if the recombination with loxP-RFP_lox2272 was successful. The expected band size is around 470bp. But no bands were observed.

Figure D: Agarose gel images of the loxP-lox2272 strategy

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REVIEW
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Zebrafish Is a Powerful Tool for Precision Medicine Approaches to Neurological Disorders

Katarzyna Ochenkowska[1,2], *Aveeva Herold*[1,2] *and Éric Samarut*[1,2,3]*

[1] Centre de Recherche du Centre Hospitalier de l'Université de Montréal, Montreal, QC, Canada, [2] Department of Neuroscience, Université de Montréal, Montreal, QC, Canada, [3] Modelis Inc., Montreal, QC, Canada

Personalized medicine is currently one of the most promising tools which give hope to patients with no suitable or no available treatment. Patient-specific approaches are particularly needed for common diseases with a broad phenotypic spectrum as well as for rare and yet-undiagnosed disorders. In both cases, there is a need to understand the underlying mechanisms and how to counteract them. Even though, during recent years, we have been observing the blossom of novel therapeutic techniques, there is still a gap to fill between bench and bedside in a patient-specific fashion. In particular, the complexity of genotype-to-phenotype correlations in the context of neurological disorders has dampened the development of successful disease-modifying therapeutics. Animal modeling of human diseases is instrumental in the development of therapies. Currently, zebrafish has emerged as a powerful and convenient model organism for modeling and investigating various neurological disorders. This model has been broadly described as a valuable tool for understanding developmental processes and disease mechanisms, behavioral studies, toxicity, and drug screening. The translatability of findings obtained from zebrafish studies and the broad prospect of human disease modeling paves the way for developing tailored therapeutic strategies. In this review, we will discuss the predictive power of zebrafish in the discovery of novel, precise therapeutic approaches in neurosciences. We will shed light on the advantages and abilities of this *in vivo* model to develop tailored medicinal strategies. We will also investigate the newest accomplishments and current challenges in the field and future perspectives.

Keywords: precision medicine, neurological disorders, zebrafish, functional genomics, drug discovery

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Sepand Rastegar,
Karlsruhe Institute of Technology (KIT), Germany
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***Correspondence:**
Éric Samarut
eric.samarut@umontreal.ca

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INTRODUCTION

Precision medicine is one of the so-called "hot topics" in applied sciences, modern biomedicine, and biomedical studies. However, the term "precision medicine" is an umbrella for a vastness of definitions that could make its understanding complex, imprecise and somehow confusing. As a generally acknowledged definition, precision medicine, also known as personalized medicine, is a form of medicine that considers individual variability in genes, environment, and lifestyle to prevent, diagnose or treat a disease. As per the Centers for Disease Control and Prevention CDC, "*precision medicine helps doctors find unique disease risks and treatments that will work best for patients*." Some may think that personalized medicine is an invention of the 21st century. However,

in the light of these general definitions, this facet of medicine and human sciences is not new. Indeed, since the dawn of time, physicians have always tailored their medical recommendations to individual factors such as age, gender, and other patients' lifestyle specificities. First reports of adapting medicine to an individual's health status are found in the history of ancient Egypt, where the medicine was divided into categories by different body parts (Visvikis-Siest et al., 2020). Then, with the rise of modern medicine in the 20th century, precision medicine relied on more accurate molecular clues such as rhesus factors defining blood groups (Klein et al., 2015). Thus, it quickly became practical to group patients based on their blood type to improve successful blood transfusions. The *personalized* aspect of medicine became more tangible at the beginning of the 21st century following human genome sequencing. Indeed, it is now possible to correlate the genetic fingerprint of individuals to their general health and treatment responsiveness (Brittain et al., 2017; Carrasco-Ramiro et al., 2017). This is the basis of the modern definition of precision medicine that aims to improve diagnosis and prognosis and tailor the medication to an individual based on genetic variations.

Because a simple definition of precision medicine cannot quickly be drawn, it seemed essential to us to start this review by determining three main pillars of precision medicine we will be discussing here: (1) Predicting disease susceptibility, progression, and improving diagnosis, (2) Accelerating drug discovery; (3) Predicting treatment-responsiveness and eliminating trial-and-error inefficiencies of current medical plans. Importantly, these main missions of precision medicine can be applied to a broad list of biomedical disciplines, including oncology (Krzyszczyk et al., 2018; Gambardella et al., 2020; Malone et al., 2020), immunology (Boyd et al., 2017; Ballow and Leiding, 2021; Distler et al., 2021), and neurological disorders (Freudenberg-Hua et al., 2018; Schneider and Alcalay, 2020; Striano and Minassian, 2020).

Although we have just stated that precision medicine is not a novel aspect of science, research investigating the correlation of genetic factors to medical outcomes is still in its infancy. Such translational research relies on developing predictable, reproducible, and reliable animal models in which genotype to phenotype correlations can be accurately assessed. Moreover, these models must be amenable to pharmacological studies and drug screening approaches. Zebrafish is a well-known model for investigating biological issues, particularly central nervous system (CNS) development (Blader and Strahle, 2000; Schmidt et al., 2013). Embryos are convenient for genetic manipulations, including CRISPR-CAS9 genome engineering (Hwang et al., 2013) and the development of CNS structures can be followed *in vivo* at a single-cell resolution thanks to an extensive repertoire of available transgenic lines (Park et al., 2000; Zerucha et al., 2000; Shin et al., 2003; Kimura et al., 2006). Zebrafish have an integrated nervous system, and the brain of a zebrafish larvae already contains homologous brain structures to those found in mammals (Vaz et al., 2019; Corradi and Filosa, 2021) as well as equivalent cellular and synaptic networks and functions (Kaslin and Panula, 2001; McLean and Fetcho, 2004; Filippi et al., 2010; Panula et al., 2010). Furthermore, the zebrafish embryo or larva shows a complex behavioral repertoire as early as a few days post-fertilization (Tegelenbosch et al., 2012). Finally, being favorable to preclinical drug discovery, zebrafish is a model of choice for pharmacological studies.

In this review, we will limit ourselves to the development of precision medicine approaches in the context of neurosciences. Particularly, we will discuss the predictive power of zebrafish in the discovery of novel, precise therapeutic approaches in this framework. We will shed light on the advantages and abilities of this *in vivo* model to develop tailored medicinal methods. Mainly, we will discuss how it can participate in the mission of the three pillars of precision medicine we described above. We will also investigate the newest accomplishments and current challenges in the field and future perspectives it could offer.

USING ZEBRAFISH TO PREDICT NEUROLOGICAL DISEASE SUSCEPTIBILITY AND PROGRESSION AND IMPROVE DIAGNOSIS

Unraveling Pathogenic Molecular Mechanisms by Functional Genomics Using Zebrafish

The study of the mechanisms that predispose, cause, or participate in the development of neurological pathologies can be enlightened through functional genomics approaches using adequate experimental models. As the name suggests, *functional genomics* aims at deciphering the function of genes and their role in a genuine biological system (healthy and unhealthy). A few decades ago, complete genome sequencing provided a framework for comprehensively investigating biological processes (Hocquette, 2005; Bunnik and Le Roch, 2013). Functional genomics integrates molecular biology and cell biology studies to explore the whole structure, process, and regulation of genes of interest to examine the function of a myriad of genes with unknown parts. Even if the causative gene is identified for many genetic diseases, the molecular pathogenic substratum often remains unknown. This issue is more critical in the context of rare genetic neurological disorders since they are understudied and not well-understood. It is essential to unravel the molecular mechanisms underlying the disease phenotype as they could identify actionable targets for further therapeutic development.

The zebrafish model is an excellent experimental approach for such functional investigations as a valuable scientific tool. Indeed, the latest improvements in bioengineering techniques allow researchers to study the functions of genes and the impact of their mutations directly *in vivo* in zebrafish larvae. As vertebrates, zebrafish, and human genomes show a high homology, about 80% of genes associated with diseases in patients are conserved in zebrafish (Kalueff et al., 2014). Notably, many CNS-related disorders have been successfully modeled in the past and some recent reviews have compiled an exhaustive list of zebrafish models of Amyotrophic Lateral Sclerosis (ALS) (Braems et al., 2021), Hereditary Spastic Paraplegia (HSP) (Naef et al., 2019; Quelle-Regaldie et al., 2021a,b), Epilepsy

(Rosch et al., 2019; Gawel et al., 2020), Autism Spectrum Disorder (ASD) (Meshalkina et al., 2018; de Abreu et al., 2020), Alzheimer's Disease (AD) (Saleem and Kannan, 2018), Parkinson's Disease (PD) (Unal and Emekli-Alturfan, 2019; Najib et al., 2020), Huntington's and Prion-related diseases (Wang et al., 2021), Serotonin syndrome (SS) (Stewart et al., 2013), and Glioblastoma (Reimunde et al., 2021). In this context, our group pioneered the generation of several models of CNS genetic disorders, caused by mutations in *gabra1* (Samarut et al., 2018), *gabrg2* (Liao et al., 2019), *depdc5* (Swaminathan et al., 2018), *glra1* (Samarut et al., 2019), or *gldc* (Riche et al., 2018), and these mutants display clinically-relevant phenotypes such as seizures, ataxic motor phenotypes or hypotonia. More importantly, studies at the cell network and molecular levels using these zebrafish genetic models have made it possible to highlight novel aspects of the underlying pathogenicity. For instance, mimicking DEPDC5 (DEP containing 5 domain) loss-of-function in zebrafish recapitulates critical hallmarks of brain disorders caused by mutations in this gene, such as epileptiform discharges and exacerbated mTOR signaling (de Calbiac et al., 2018; Swaminathan et al., 2018). However, a closer examination of neural cell networks *in vivo* revealed a drastic reduction in the number and complexity of inhibitory synapses in the brain of *depdc5-/-* zebrafish larvae compared to their siblings. More excitingly, pharmacological studies showed that this phenotype is not caused by a defect in the classical function of the *DEPDC5* gene (e.g., mTOR inhibitor) but is rather mTOR-independent. This study, therefore, opens a yet undiscovered aspect of DEPDC5 biology and related brain disorders. In the same line of thought, modeling loss-of-function in a gene involved in glycine breakdown (glycine decarboxylase, *GLDC*) in zebrafish leads to an increase in the accumulation of glycine in tissues and to premature death, as is the case in corresponding mouse models (Narisawa et al., 2012; Pai et al., 2015; Riche et al., 2018). However, thanks to the accessibility of zebrafish embryos to quantitative molecular techniques, such as whole transcriptome sequencing, it was shown that the metabolic perturbances caused by GLDC loss-of-function in zebrafish go beyond glycine only. Indeed, molecular profiling of key amino-acid metabolites by liquid-chromatography mass-spectrometry (LCMS) in *gldc-/-* zebrafish larvae identified significant changes in the level of several branched-chain amino acids (Riche et al., 2018). Interestingly, other disorders caused by an accumulation of these branched-chain amino acids have been described, and this calls for new potential cross-comparisons between neurometabolic diseases. Interestingly, such exploratory molecular profiling assays *in vivo* in zebrafish models of genetic disorders can also help identify potential novel disease biomarkers that can further be validated in the patient populations.

Finally, it is worth mentioning that zebrafish can also be used to unravel fundamental aspects of pathologies without necessarily necessitating the use of genetic models. Indeed, thanks to its optical transparency and an extensive repertoire of available transgenic lines (Park et al., 2000; Zerucha et al., 2000; Shin et al., 2003; Kimura et al., 2006), zebrafish are convenient to follow, *in live*, brain activity under specific pharmacological exposures. For example, Diaz-Verdugo et al. studied the respective roles of non-neuronal glial cells vs. neurons in the brain state transition leading to epilepsy brain seizures (Diaz Verdugo et al., 2019; Rosch et al., 2019). To do this, they elegantly utilize zebrafish larvae to record the activity of both neurons and glial cells following exposure to a proconvulsant GABA-antagonist (pentylenetetrazol or PTZ). Surprisingly, their work revealed a robust and widespread activation of a glia network *before* the generalization of neuronal activity, the latter being characteristic of generalized epileptic seizures. They showed that whereas glial activation before brain seizure seems to reduce synchronous neuronal activity, a collapse of the glial homeostatic regulation may precipitate a generalized release of glutamate in the extracellular space leading to a generalized seizure. This provides a new perspective on seizure spreading in the brain and opens a new door to therapy development targeting non-neuronal cells.

Thanks to its pharmacological and genetic accessibility, the use of see-through zebrafish larvae is a powerful catalyzer for discovering novel pathogenic mechanisms associated with neurological disorders. Such findings are instrumental for accelerating the precision medicine mission that aims to predict disease susceptibility and prevent its progression. Although these studies strongly advocate for the translational potential of CNS investigations in zebrafish, one must keep in mind that there are some important differences in the development of certain brain areas between zebrafish and mammals. Many structures in the zebrafish brain can be considered neuroanatomical similar to mammalian ones, however, they somehow display morphological differences in their development (Friedrich et al., 2010; Cheng et al., 2014). Moreover, as for many non-primate animal models, zebrafish is lacking a canonical hippocampus or cortex. As a result, these developmental and/or morphological discrepancies must be taken into account when modeling brain diseases in zebrafish.

Validating Genetic Variants Associated With Neurological Disorders Using Zebrafish

One of the main current challenges in precision medicine is improving and accelerating disease diagnosis. Next-generation sequencing radically changed biomedicine by identifying loci, genes and associated mutations involved in specific diseases (Phan et al., 2006). These new genetic data are instrumental for discovering the etiology of human diseases, and the number of new disease-causing genetic variants exploded (Koboldt et al., 2013). However, the clinical relevance of genetic information can be limited by the lack of a precise functional characterization. Thus, there is a need to validate the role played by a specific mutation in a simple biological system to infer a pathogenic role. This is particularly relevant in the case when Variants of Unknown Significance (VUS) are identified in patients. These genetic variations for which we do not know the molecular and physiological consequences represent a significant dilemma in genetic diagnosis and genetic counseling (Alosi et al., 2017). Indeed, according to the American College of Medical Genetics and Genomics (ACMG) guidelines, VUS should not be used in

clinical decision-making (Richards et al., 2015). Unfortunately, VUS can be predominantly found in sequencing-based clinical genetic tests. For example, in a cross-sectional study of 164 epileptic patients followed by an epileptologist at a Canadian tertiary care centre's epilepsy clinic, VUS accounted for more than half of the genetic test results (Li et al., 2022). Based on these unpublished data, it is worth noting that if only 10% of the VUS identified could be functionally validated (a rather conservative estimate), this would potentially increase by 70% the overall positivity yield of the genetic testing in this clinical study. Thus, the clinical advantages of genetic testing can be multiplied *via* the simultaneous development of approaches that aim at functionally validating VUS.

Importantly, VUS identified from a targeted genetic panel (rather than from exploratory Whole-Exome-Sequencing) is often a better fit with the patient's clinical presentation. In those cases, further *in vitro* and *in vivo* functional testing could help confirm, exclude, or guide clinicians toward a diagnosis. Functional characterization is usually performed in research laboratories, and different levels of functional assessment can be achieved from *in vitro* (i.e., recombinant enzyme activity), *in cellulo* (i.e., target gene expression, cellular phenotype) to *in vivo* (i.e., tissue homeostasis and function, behavior). The latter encompassing a higher degree of complexity necessitates fast and complementary *in vivo* approaches that can remain expensive and time-consuming. The development of a platform for the functional characterization of VUS has been shown very successfully in the field of oncology, particularly breast cancers associated with mutations in *BRCA* genes, leading to a significant improvement in the clinical management of cancer patients (Guidugli et al., 2014; Woods et al., 2016; Federici and Soddu, 2020). Another example is the different pathogenic variants in the gene *PALB2* associated with varying levels of risk for breast, ovarian and pancreatic cancers (Boonen et al., 2020). Using a combination of complementary *in vitro* assays, researchers can assess the impact of specific *PALB2* VUS, at the level of individual patients, on the function of the protein in DNA repair, cell cycle regulation and the control of cellular levels of reactive oxygen species (Boonen et al., 2020). This functional characterization of VUS can be valuable for predicting cancer risk and anticipating treatment-responsiveness to cancer therapy for each patient.

Remarkably, simple animal models (i.e., worms, flies, fish) can be sophisticatedly employed to bridge the gap between genetic diagnosis and functional studies. Being compatible with the latest mutagenesis techniques and convenient for deciphering basic pathological mechanisms caused by gene mutations, they can open new avenues for VUS functional characterization. Depending on the class of the genetic mutation of interest to be studied, different functional characterization approaches can be followed using zebrafish (**Figure 1**). Simplistically, primary pathogenic mechanisms can be divided into a toxic gain of function (GoF) or deleterious loss of function (LoF). Predictably, diseases with a LoF mechanism are inherited in an autosomal recessive manner or an X-linked recessive manner. On the contrary, conditions due to a toxic GoF mechanism are usually inherited autosomal dominant. However, non-exclusive pathogenic LoF and GoF mechanisms can coexist, thus complexifying the study of their pathogenicity in a standardized fashion.

Due to external fertilization, zebrafish embryos can be microinjected at the one-cell stage with *in vitro* transcribed mRNAs for overexpressing a construct of interest. This technique has been exploited for the expression of patient-specific mutations in genes associated with neurological disorders such as Amyotrophic Lateral Sclerosis (Armstrong and Drapeau, 2013) or small-fiber neuropathy (Eijkenboom et al., 2019) as well as for non-neurological conditions such as sinus node dysfunction and atrial fibrillation (Hoffmann et al., 2019). In these studies, clinically relevant phenotypes are assessed in few-day-old larvae, such as swimming behavior, touch-evoked motor response, sensory neurite development or electrophysiologic hallmarks. This transient expression method does not require complex genetic manipulation and can be carried out on large scales and in a short period. However, the experimental variability associated with the manual microinjection of hundreds of embryos and the short half-life of mRNA in the embryo can complicate the interpretation of negative results. To circumvent this problem, other studies took advantage of transposase-mediated stable genomic integration techniques such as Tol2 (Urasaki et al., 2008; Suster et al., 2009) or Sce-I (Hoshijima et al., 2016) random mutagenesis (**Figure 1**). Researchers generated stable transgenic lines expressing the wild-type or mutant version of a gene of interest, such as the ALS-causative G348C mutation in TDP-43 (Lissouba et al., 2018) or the C1315Y mutation in *COL2A1* associated with lethal fetal skeletal dysplasia (Zhang et al., 2021). Moreover, this technique allows using specific promoter sequences to regulate the expression of the transgene in a tissue- or time-specific fashion. Although this approach has the advantage of not being limited to observing effects solely in the early embryo, it presents one main limitation. Indeed, phenotypes observed upon the stable integration of mutant versions of genes must be carefully compared to their wild-type form. Because of the random nature of these transgenesis techniques, comparing wild-type and mutant allelic versions of a gene of interest that have not been integrated at the same genomic locus and potentially in different copy numbers can be seen as inaccurate. Moreover, the random integration of a transgene of interest remains simulated and cannot accurately be compared to a genuine endogenous expression.

Proving the pathogenicity of loss-of-function mutations can be less challenging, especially in the case of deleterious mutations such as sudden stops or large genomic deletions. In these cases, transient knockdown by morpholino microinjection has been used for years to quickly assess the phenotypic consequence of a specific loss-of-function for *CHD2* in epileptic encephalopathy (Galizia et al., 2015), *CAPN1* in the context of Hereditary Spastic Paraplegia (Gan-Or et al., 2016), ABCC6 in Pseudoxanthoma elasticum (Van Gils et al., 2018), *SBDS* in Shwachman–Bodian–Diamond syndrome (Venkatasubramani and Mayer, 2008), or *VARS* in epilepsy (Siekierska et al., 2019). However, the morpholino-knockdown approach is transient and limited to the first few days of development, and the specificity of morpholinos has also been the subject of debate recently (Stainier et al., 2017). Particularly, strict guidelines have been drawn for the

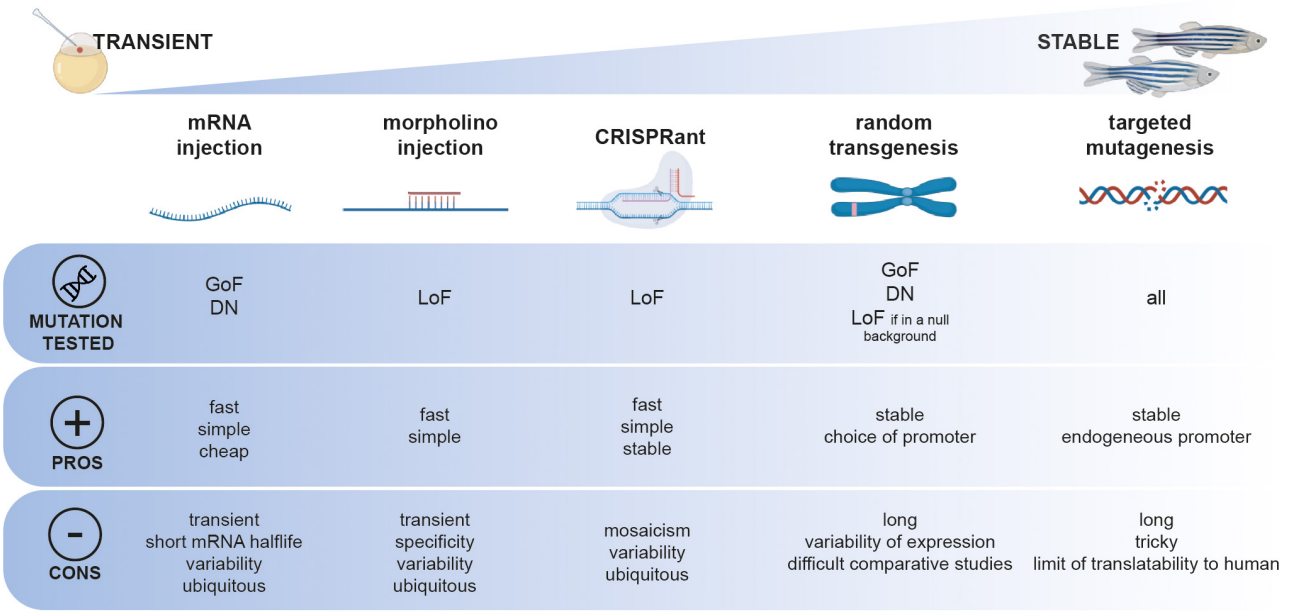

FIGURE 1 | Functional toolbox to study the function of genetic mutation in zebrafish. Different approaches, from transient to stable can be employed and can be used depending on the type of mutation to be tested (GoF, gain-of-function; DN, dominant-negative; LoF, loss-of-function).

interpretation of morpholino-based assays in zebrafish (Stainier et al., 2017). To overcome these drawbacks, it is now well-admitted in the zebrafish community that a targeted-mutagenesis assay must be preferred, such as Zinc Finger Nucleases (Doyon et al., 2008), TALEN (Hwang et al., 2014), or the popular CRISPR/CAS9 (Hwang et al., 2013; Ran et al., 2013). Targeted mutagenesis tools can also be used for rapid screening in the F0 injected larvae referred as "CRISPRant" using a combination of multi-loci guide RNAs (Kroll et al., 2021). Upon confirmation of a particular phenotype, these injected F0 CRISPRant can be raised and further screened as founders for establishing a stable mutant line. These genetic models can also be used as null *in vivo* genetic backgrounds in which specific genetic variants of the gene of interest can be transiently or stably expressed (mRNA microinjection vs. transposase-mediated random transgenesis). If the wild-type variant can rescue a particular phenotype in a quantitative assay, then it is possible to test the rescuing potential of novel VUS. In that case, the pathogenicity of a specific variant is attributed to the lack of phenotypic rescue. A recent study by Li et al. (2017) from the Harvard Medical School performed a large-scale functional screening of rare genetic variants in the Interferon Regulatory Factor 6 (*IRF6*) gene, potentially associated with orofacial cleft syndromes. They took advantage of a very early-onset embryonic phenotype caused by *irf6* knockout, that is, the improper development of the embryonic epithelium during epiboly, a process occurring only a few hours post-fertilization in zebrafish. The authors used this early rescue assay to test the protein functions of more than 30 human *IRF6* missense variants. Remarkably, they assessed the ability of each genetic variant to rescue the early epiboly defects described in *irf6*-/- embryos through mRNA microinjections at the one-cell stage. Interestingly, when comparing their functional testing results with computational pathogenicity prediction systems (PolyPhen-2 and SIFT), they confirmed the pathogenicity of variants classified as "pathogenic" but found discrepancies in interpretation for about 50% of the variant classified as "likely pathogenic." These results reinforce the idea that a functional validation of VUS is essential before inferring a level of pathogenicity to specific genetic variants.

Finally, the latest advances in targeted genetic engineering, especially using CRISPR-CAS9, allow researchers to directly mimic patient-specific missense mutations onto the endogenous zebrafish gene. Indeed, homologous recombination events can occur by adding a nucleic acid donor template to the CRISPR cocktail to be microinjected. Although, due to technical limitations, the overall efficiency of this knock-in application remains much lower as compared to the generation of knockout mutations, the generation of such patient-specific genetic avatars has been accomplished in TDP43/ALS (Armstrong et al., 2016), in *FBN1* in various heritable connective tissue disorders (HCTD) (Yin et al., 2021), RPS14 in myelodysplastic syndrome (MDS) (Ear et al., 2016). One of the limitations of this approach is that the protein residue that is the subject of the mutation may not be conserved in zebrafish. Additionally, it is possible that although an orthologous gene is present in the zebrafish genome for a particular study, specific missense mutations may not lead to the same effects in a human or fish protein. An alternative method would necessitate recombining, at the endogenous locus, the whole human coding DNA as a transgene, but such genetic engineering would be time-consuming. Interestingly, a recent work using nematode worms describes such a "humanized"

functional assay by developing an *in vivo* platform for screening the pathogenicity of VUS in the *STXBP1* gene associated with epileptic syndromes (Zhu et al., 2020). In this study, mutations were introduced by CRISPR-Cas9 and modeled using *Caenorhabditis elegans* to mimic and investigate the pathogenicity of gene variants. This illustrates, once again, how simple *in vivo* assays could quickly and accurately determine if a VUS is pathogenic or benign and how this could be applied to zebrafish.

In summary, *in vivo* zebrafish studies can provide an additional line of biological evidence to bridge the gap between variant identification and their pathogenic classification. Zebrafish can be considered an attractive multi-assay platform to characterize the pathogenicity of specific genetic mutations. However, the experimental approach must be defined according to the type of mutation apprehended (loss-of-function vs. gain-of-function; **Figure 1**). Specific technical limitations may hinder the standardization of these functional approaches *in vivo*.

USING ZEBRAFISH TO ACCELERATE DRUG DISCOVERY

Drug discovery is a complex and lengthy process that entails years of meticulous planning, from the initial discovery of an active ingredient (drug-like compound) to the development process, which includes testing in animal models and finally in humans (Zon and Peterson, 2005). On average, it takes 10–15 years and US$2.6 billion for an active ingredient to reach the bedside (Seyhan, 2019) and this is primarily due to the failure of several candidate compounds at various stages of the drug discovery timeline (Bhusnure et al., 2015). Although *in vitro* and *in vivo* mammalian models are used to lower the cost and time of drug discovery, *in vitro* studies are less human-translatable and mammalian models make the entire process time-consuming, expensive, and laborious. Zebrafish can bridge the gap between *in vitro* studies and rodent models due to easy maintenance, cost-effectiveness, and reduction in the number of animals employed in regulatory testing according to the 3Rs (replace, reduce, refine) (Fleming and Alderton, 2013; Geisler et al., 2017). They also enable an early prediction of *in vivo* toxicity and safety data, which reduces the likelihood of drug failure later on. This section of the review will discuss how zebrafish can be used as a predictive preclinical model to accelerate various stages of drug development, from early discovery to preclinical development (**Figure 2**).

Early Drug Discovery

The end goal of the early drug discovery phase is to identify an active compound that has the potential to develop as a drug. Zebrafish can be utilized in the early stages of drug development using two primary methodologies: target-based and phenotype-based approaches.

The starting point of target-based approaches is a defined molecular target that is hypothesized to have an essential role in disease. Such target-centric methods have been the dominant approach to drug discovery in the pharmaceutical industry. However, the process of target *validation* is complex and associated with a high degree of uncertainty and failure. Thus, target identification is critical in this process, and selecting a relevant target requires a greater understanding of the disease's pathophysiology and molecular process (Gashaw et al., 2012). As zebrafish have been successful in mimicking a plethora of neurological disorders (Ramesh et al., 2010; Khan et al., 2017; Fontana et al., 2018; Gawel et al., 2020; Razali et al., 2021; Wang et al., 2021), it can pave the way for the discovery of such novel drug targets. One classical approach to target identification in zebrafish has been the use of morpholino-oligonucleotides but as said previously, their popularity and the confidence in their specificity has declined. Only a limited number of studies to date have used novel targeted-mutagenesis techniques such as CRISPR/Cas9 to generate target discovery studies in the neuroscience field. However, it is expected that a growing number of studies will be reported soon (Rubbini et al., 2020) as shown by the example of target identification in a *C3orf70* knockout zebrafish mutants with impaired circadian rhythm and altered light-dark neurobehaviors (Ashikawa et al., 2019) in which the *C3orf70* gene was reported to be a shared target of *Neurog1/2* and *Asc11*. As a result, *C3orf70* mutations may be linked to neurodevelopmental and neuropsychiatric diseases in these brain locations and could be exploited as a therapeutic target (Ashikawa et al., 2019). Target identification can also arise from exploratory transcriptomics investigation on a genetic zebrafish model for a particular disease. For instance, our group developed a zebrafish model of glycine encephalopathy carrying a mutation in a glycine metabolism enzyme (*GLDC*: glycine decarboxylase) (Riche et al., 2018). By whole transcriptome analysis of larval mutant brains, our study revealed changes in essential genes regulating the synaptic clearance of glycine, such as glycine transporter 1 (*glyt1*). Such a reduction of expression of this transporter exacerbates the accumulation of glycine at the synapse, thus the neurometabolic phenotype. Following target identification, the next step is to validate its involvement in the pathogenicity of the disease of interest to confirm its relevance and pave the way for defining essential properties that future hit compounds targeting it must fulfill. In the example of glycine encephalopathy, we demonstrated that boosting the expression of *glyt1* in mutant zebrafish embryo by mRNA microinjection was able to rescue the early motor phenotype associated with the mutation (Riche et al., 2018). Therefore, *glyt1* appears as an interesting novel target in the context of this disease. The use of zebrafish for target validation is also exploited by several contract research organizations (CROs) that propose to perform standardized functional assays on a target of interest (BioBide, ZeClinics, INVENesis, *InVivo* Biosystems). However, target-based screening for CNS disorders is more challenging due to the multifactorial nature of many neurological diseases.

Unlike target-based techniques, *in vivo* phenotype-based drug discovery allows the identification of compounds that modify the disease phenotype with no prior knowledge of a particular target. Notably, such target-agnostic approaches in the whole organism can later identify novel therapeutic targets. Currently, zebrafish is widely used as an *in vivo* phenotypic screening platform and has led to the development of several successful therapeutics.

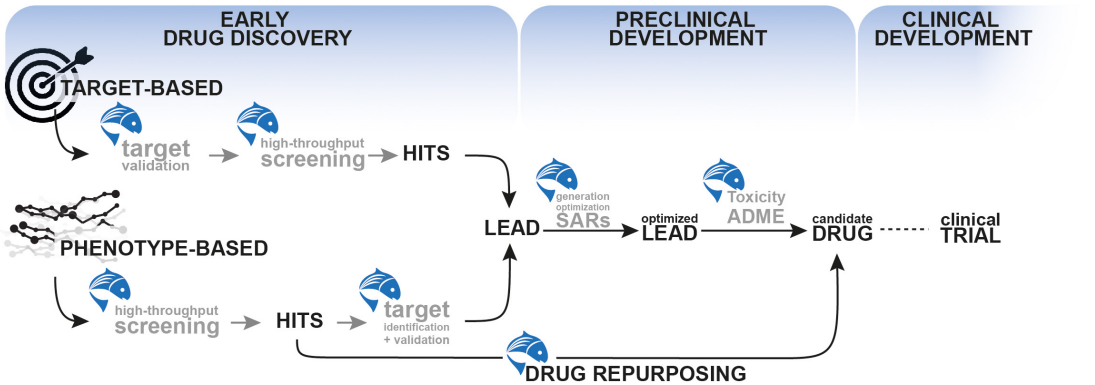

FIGURE 2 | The use of zebrafish as a predictive preclinical model to accelerate various stages of drug development, from early discovery to preclinical development.

As a first step, phenotypic screening aims to identify a so-called "hit" compound with the desired effect on the phenotype screened. Thanks to its small size, zebrafish larvae can be placed in multi-well plates for screening the impact of various molecule libraries (including bioactive compounds, commercially available chemical libraries, and natural compounds) on a specific phenotype such as behavioral tracking, gene expression assay, or fluorescent reporter studies (Mills and Gallagher, 2017; Deakin et al., 2019; Abdelmoneim et al., 2020). Simple motor phenotypes can be assessed, such as a simple photo motor response assay using zebrafish embryos that allows screening for different profiles of psychotropic compounds in a high-throughput fashion (Kokel et al., 2010). In a *sod1*-mutant zebrafish model of Amyotrophic lateral sclerosis, McGown et al. (2016) developed a high throughput screening assay using a fluorescence-based readout of neuronal stress. Another commonly used behavioral assay is the increased motility response induced by exposure to Pentylenetetrazole (PTZ), a pro-convulsant drug (Baraban et al., 2005; Winter et al., 2008). Such an assay has been used to identify anti-seizure compounds *in vivo* in zebrafish. For instance, Baxendale et al. (2012), screened approximately 2,000 compounds and identified 46 hit molecules that suppressed PTZ-induced seizure phenotype in zebrafish larvae. Moreover, Kim and colleagues performed a screen of 1,403 bioactive compounds using an *in vivo* whole-organism screening assay by imaging dopaminergic neurons of larval zebrafish in a high throughput manner. In short, the authors used a transgenic zebrafish model in which they induced dopaminergic neuron loss. Using an automated imaging microscope, they screened the effect of each compound on the survival of this neuronal population *in vivo*. Their study identified several hit molecules that significantly protected dopaminergic neurons in this assay (Kim et al., 2022). These examples show how zebrafish can identify hit compounds unbiasedly using phenotypic screening. Once a list of hit molecules is in hands, the next step is to further evaluate them based on more selective screening criteria to select the "lead" molecules that are more likely to be therapeutically valuable (hit-to-lead phase, **Figure 2**). Although zebrafish have been extensively used for hit identification using high-throughput screening, it is less frequently used to further characterize and optimize lead candidate compounds. However, zebrafish genetic models could be used to correlate phenotypic readouts with target binding and could therefore be a convenient platform for the optimization of lead candidates (lead expansion phase). One can imagine that the classical way of thinking surrounding more advanced therapeutic development necessitates the use of mammalian models. However, in the next section, we will see that zebrafish can autonomously bridge the gap between bench and bedside.

Drug Repurposing

As we discussed previously, zebrafish are convenient for phenotypic screening, and the effect of thousands of compounds can be tested on various disease-relevant phenotypes. Such molecule libraries can contain already approved drugs for which a novel therapeutic indication could be unveiled. Repurposable drugs have a low risk of failure since their toxicology profiles have been thoroughly determined in previous clinical studies and are known to be safe in humans (Ayyar and Subramanian, 2022). Using zebrafish, phenotypic screening of over 3,000 commercially available FDA-approved drugs has been conducted on a genetic zebrafish model of Dravet syndrome, a severe infantile epilepsy (Baraban et al., 2013; Dinday and Baraban, 2015; Sourbron et al., 2016). In this work, the authors identified several 5-HT serotonin-receptor agonists as being able to suppress spontaneous seizures in mutant larvae. The phenotypic assay was conducted on a behavioral seizure assay and then confirmed using brain electrographic recordings (Baraban, 2013). Based on these results, they jumped straight from tank to bedside by treating pharmacoresistant Dravet syndrome patients with lorcaserin, a clinically approved serotonin receptor agonist (compassionate use) (Griffin et al., 2017). Interestingly, they observed reductions in seizure frequency and severity, thus proving the relevance of targeting the serotonin receptor pathway in Dravet syndrome (Griffin et al., 2017). Among the hit compounds they identified, clemizole, a classical antihistaminic agent, was found particularly efficient in preventing seizures. Further protein binding profiling showed that clemizole could act as a serotonin receptor antagonist, and a spin-off biotech

(EpyGenix Therapeutics) is currently developing clemizole (EPX-100) and derivatives (EPX-101, EPX-102, and EPX-103) for the treatment of Dravet syndrome.

Another example of a successful drug-repurposing screen is the work of Patten and colleagues, who performed a chemical screening of 3,850 small molecules on *C. elegans* models of Amyotrophic Lateral Sclerosis (ALS). This initial screen identified 13 hits compounds further tested in transgenic zebrafish expressing mutant TDP-43. The authors assessed the effects of these hit molecules on multiple phenotypes from swimming motility, known to be reduced in mutant-TDP43 expressing transgenic fish, orphan neuromuscular synapses and electrophysiology recordings of synaptic transmission (Patten et al., 2017). They showed that one hit molecule, pimozide, was particularly effective at alleviating these phenotypes. They further confirmed the neuroprotective effect of pimozide on two other zebrafish ALS models expressing FUS(R521H) and SOD1(G93A) based on motor phenotypic readouts (swimming response, swim duration, distance swam, and maximum swim velocity). Finally, after confirming the positive effect of pimozide on stabilizing the neuromuscular neurotransmission in a genetic mouse model of ALS, the authors initiated a short randomized controlled trial of sporadic ALS patients. Remarkably, this small-scale phase 2A trial demonstrated stabilization of motility and evidence of target engagement at the neuromuscular junction in ALS patients (Patten et al., 2017). A phase II randomized, placebo-controlled, double Blinded, multi-centered phase 2B clinical trial is ongoing to confirm the positive effect of pimozide in 100 ALS patients (NCT03272503). Interestingly, as a neuroleptic, pimozide specifically targets dopamine D2 receptors. Still, the authors showed that its effect on neuromuscular junctions relies on the antagonism of T-type Ca2 + channels, one off-target property of pimozide. Thus, the main accomplishment of this work does not solely rely on the discovery of a new therapeutic compound for ALS (the first such accomplishment in nearly two decades) but also on the unveiling of a novel pathogenic mechanism that can be further harnessed for the development of new therapeutics targeting T-type calcium channels.

These studies illustrate how zebrafish can be effectively used in early drug discovery processes from hit identification, hit-to-lead selection and target discovery and validation (**Figure 2**).

Preclinical Studies

Once a lead compound is identified from the early drug discovery phase, it usually undergoes optimization through structure-activity relationship (SAR) profiling. These preclinical studies aimed to refine the lead molecule's structure and apprehend the lead molecule's safety *in vivo* while improving its adsorption properties (Temml and Kutil, 2021). There is an interest in incorporating these lead optimization essays as early as possible during the drug discovery process to increase the chance of success while reducing the costs associated with failure later on. Zebrafish is highly amenable to studying such structure-activity relationships (SARs) and therefore participate in predicting the biological activity of compounds based on their molecular structure and improve the development of structural analogs with better activity. Indeed, several SAR studies have been performed in zebrafish, where the effect of different structural analogues (first obtained from *in vitro* studies, such as molecular docking or ligand-receptor binding studies) is tested on zebrafish behavioral phenotypes as readouts. Coming to the example of clemizole that has been identified using zebrafish chemical screening for Dravet syndrome (discussed above), SAR studies have been conducted on 28 newly synthesized analogues of clemizole with varying 5-HT2R binding affinities (Griffin et al., 2019). The authors identified three analogs, specifically binding to 5-HT2B receptors, exerting a potent suppression of convulsive swim behavior and electrographic seizure activity in *scn1lab* zebrafish models of Dravet syndrome (Griffin et al., 2019).

Another example is the use of zebrafish to perform SAR studies for an antipsychotic compound in the context of schizophrenia (Hellman et al., 2020). In this study, Hellman et al. aimed at developing analogs of N-Desmethylclozapine (NDMC), the primary metabolite of clozapine that is the leader of the so-called "atypical" or second-generation antipsychotics. NDMC can act as muscarinic M1 receptor agonists, and this activity is associated with improvement in cognitive functioning in patients. However, NDMC failed phase 2 clinical trials on schizophrenic patients. Thus, the development of NDMC analogs with enhanced M1 receptor agonist functions might be promising. Using *in vivo* behavioral response profiles in zebrafish, they evaluated the antipsychotic efficacy of several NDMC analogs they developed and identified one of them that demonstrated antipsychotic activity similar to clozapine, including M1 agonist activity. Thus, thanks to this SAR study using *in vivo* phenotypic zebrafish readout, they identified one interesting NDMC analogue suitable for further development as an antipsychotic compound with potential procognitive activity (Hellman et al., 2020).

The next step after optimizing leads is to assess the toxicity profile of the lead molecule. Indeed, many lead compounds fail later in the development phase due to toxicity and efficacy concerns. Mainly, neurotoxicity is one of the significant attritions in drug development. Here again, zebrafish can be used as a predictive preclinical model to rapidly eliminate hazardous compounds and prioritize compounds for further clinical studies (McGrath and Li, 2008). The most common neurotoxic endpoints are alterations in zebrafish neurobehavioral when exposed to various toxins or chemicals. Several neurotoxicity assays are performed using zebrafish behavior phenotypes as readouts obtained from locomotor tests, photo motor tests, touch response, and acoustic tests (Polaka et al., 2022). The photo motor response assay, which involves the automatic tracking of larval movement in response to various lighting conditions, is widely used for neurotoxic screening (Cassar et al., 2020). For example, Knecht et al., used a larval photo motor experiment to test the neurotoxic effects of benzo[a]pyren. This ubiquitous environmental pollutant may contribute to human cancer development (Knecht et al., 2017). Other studies showed that the zebrafish embryotoxicity test [ZET, OECD236 (Busquet et al., 2014; Braunbeck et al., 2015)] could accurately predict the toxicity of known developmental neurotoxicant substances (Beker van Woudenberg et al., 2013).

Moreover, the zebrafish-based locomotor activity effectively classified well-known compounds as neurotoxic or non-neurotoxic, which were 90% identical to prior findings from mammals (Selderslaghs et al., 2013). Zebrafish locomotor activity can also be evaluated by touch-evoked response tests, which record zebrafish larvae's behavior in response to a tactile stimulus applied to the head or tail as a measure of sensory and motor integration (d'Amora and Giordani, 2018). The neurotoxic effects of insecticides such as endosulfan I and endosulfan sulfate were confirmed in zebrafish (Stanley et al., 2009). Finally, as said before, one main advantage of performing preclinical studies in zebrafish is that it benefits from combining SAR, toxicity and ADME (absorption, distribution, metabolism, and excretion) studies. ADME studies aim at studying the fate of an active substance contained in a drug after its administration in the body, including its absorption (A), distribution (D), metabolism (M), and excretion, including its metabolites (E). However, a key hurdle for this model is the ability to determine the effective compound concentration in the zebrafish and to correlate this dose with rodent and human data. Indeed, drug exposure remains constant as the larvae are immersed in bathing media containing the drug; thus, quantifying drug uptake into zebrafish larvae remains the main limitation. However, several techniques are being developed to accurately quantify compound uptake in larvae using mass spectroscopy or NMR to analyze the drug absorption and distribution, which are analyzed on whole embryos or specific organs or tissues (Zeclinic). Moreover, researchers from KU Leuven are developing analytical methods to measure the whole-body uptake of compounds in 10-day-old zebrafish larvae using ultra-high-performance liquid chromatography (UHPLC) (Kislyuk et al., 2017). Their data showed that a single zebrafish could be used to study the whole-body uptake of a particular drug. Then a similar methodology can be used to learn the uptake of pharmaceuticals in the brain of zebrafish and hence explore the potential of zebrafish as a predictive blood-brain-barrier model (Kislyuk et al., 2018). Moreover, using these techniques, the concentration of compounds in the incubation water can be compared to the concentration in embryos to get valuable insights into drug metabolism and excretion. In this case, metabolites, when known, may also be analyzed both in embryos and incubation water.

As a result, the shared pharmacology between zebrafish and humans makes zebrafish an important preclinical model that helps accelerate the drug discovery process at multiple levels (**Figure 2**). However, one significant limitation of the use of zebrafish as a model in early drug discovery relates to the development of the blood-brain barrier (BBB). BBB is a complex structure that represents a physical blockade for drugs to access the CNS. Thus, BBB permeability needs to be carefully taken into consideration while testing the efficiency of therapeutic compounds for brain disorders. In zebrafish, the BBB starts to form at 3 days post-fertilization but its maturation progresses until 10 dpf (Fleming et al., 2013). It is important to note that during this maturation period, during which most screening experiments are usually performed (e.g., 5 dpf), the zebrafish BBB has been described as "leaky." Thus, caution should be exercised with interpretations of BBB crossing when testing compounds on zebrafish larvae at stages when the BBB is still permeable.

USING ZEBRAFISH TO PREDICT TREATMENT-RESPONSIVENESS AND TAILOR MEDICATION

Choosing the Best Available Treatment

As discussed in the previous part of this review, zebrafish is a convenient tool for identifying new lead molecules for therapeutic purposes in neurosciences, especially epilepsy. Indeed, the similarity of brain seizures between zebrafish and humans makes this model particularly relevant for translational research perspectives. However, there is also a need to identify which drug is more likely to be efficient for a particular group of patients among the list of available treatments. Indeed, although there are 28 classical Antiepileptic Drugs (AEDs) available to epileptic patients, treatment-response is often unpredictable, and approximately one-third of patients fail to gain complete seizure control with pharmacotherapy alone (Chen et al., 2018). Moreover, determining the best AED among the 28 classical ones mostly relies on an empirical trial-and-error method from medical doctors. More importantly, it has been shown that an inappropriate first medication can have severe consequences on the efficacy of further treatments (Pawluski et al., 2018). This underlines the need to identify which AED works best for each patient as quickly as possible. In the last decades, the genetic component of many epilepsies has been unraveled, and it helped better classify the different types of epilepsies depending on their genetic etiology. In line with these discoveries, many zebrafish genetic mutants have been generated carrying mutations in different genes associated with brain seizures and epileptic syndromes (Baraban, 2007; Hortopan et al., 2010; Stewart et al., 2014; Griffin et al., 2018; Gawel et al., 2020). Our group participated in this effort by developing several zebrafish lines carrying loss-of-function mutations in epilepsy-causing genes such as *gabra1* (Samarut et al., 2018), *gabrg2* (Liao et al., 2019), *scn1lab* (unpublished), and *depdc5* (Swaminathan et al., 2018). These mutant fish undergo brain seizures either spontaneously or under stress conditions, and the treatment-responsiveness of these lines, measured unbiasedly through behavioral and/or brain activity recording readouts, recapitulates drug response in patients. This is consistent with a recent review showing that zebrafish models of Dravet syndrome are particularly reliable in pharmacological and clinical relevance (Griffin et al., 2018). The anti-seizure effect of well-known AEDs, such as valproic acid, carbamazepine, gabapentin, diazepam, lacosamide, and pregabalin, has been tested on PTZ-induced seizures in zebrafish (Baraban et al., 2005; Berghmans et al., 2007; Gupta et al., 2014). Interestingly, in these assays, many non-GABAergic drugs also prevented PTZ-induced seizures, which broadened the scope of PTZ screening in zebrafish compared to rodent models. During this study, the efficiency of AEDs has been correlated with different types of seizure-like behaviors. For instance, valproic acid, gabapentin, lacosamide and carbamazepine showed

a concentration-dependent increase in latency at all stages of seizures, which was significant for valproic acid at 300 μM to 10 mM, gabapentin at 1-10 mM, lacosamide at 100 μM to 3 mM and carbamazepine at 10–100 μM, while pregabalin failed to increase in seizure latency at all the stages compared with the control group. Other drugs showed saturated responses, such as gabapentin at > 1 mM or diazepam at 10 μM. These examples show how testing the effectiveness of different drugs on relevant fish models can help develop personalized medicine approaches, particularly in epilepsy. Indeed, by applying these standardized screening techniques to more chemical and/or genetic models of epilepsy, we could better correlate genotypes to treatment-responsiveness and translate these findings to patients by better predicting which drug is the more likely to be efficient depending on the genetic etiology of diseases. However, even at the level of a single disease such as epilepsy, the spectrum of phenotypes and the genotype-phenotype correlations are very complex since different mutations, even at the level of the same gene, can lead to different types of seizures (Johannesen et al., 2016; Kang and Macdonald, 2016; Gontika et al., 2017; Shen et al., 2017). As a result, each epileptic patient might have to be considered unique in treatment responsiveness. Thanks to the latest improvements in targeted genome editing techniques, we could foresee the future development of patient-personalized zebrafish genetic avatars that could tailor the treatment at the level of individual patients/mutations. More broadly, this emphasizes the need for better studying how variations of DNA and RNA characteristics are linked to an individual's response to medication. This is the exact definition of pharmacogenomics (PGx), another important example of the field of precision medicine, which combines pharmacology and genomics to develop effective, safe medications that can be prescribed based on a patient's genetic fingerprint. Thus, PGx has the potential to revolutionize the practice of medicine by individualizing treatment through the use of novel diagnostic approaches to predict predicting which patient will particularly benefit from a medication, which one will not respond at all, and which will experience significant negative side effects (Topic, 2008). In most cases, Single Nucleotide Polymorphisms (SNPs) are the key to a better understanding an individual's response to treatment and potential risks (Alwi, 2005; Katara, 2014). These single nucleotide changes may occur in non-coding and coding regions of the genome. This creates a broad range of genetic diversity among the population. They are also called "genetic fingerprints," which pave the way for establishing new diagnostic tools and further PGx development for individuals. Promising gene-based methods aiming at improving precision in psychotropic medication allowed the identification of specific genetic polymorphisms in

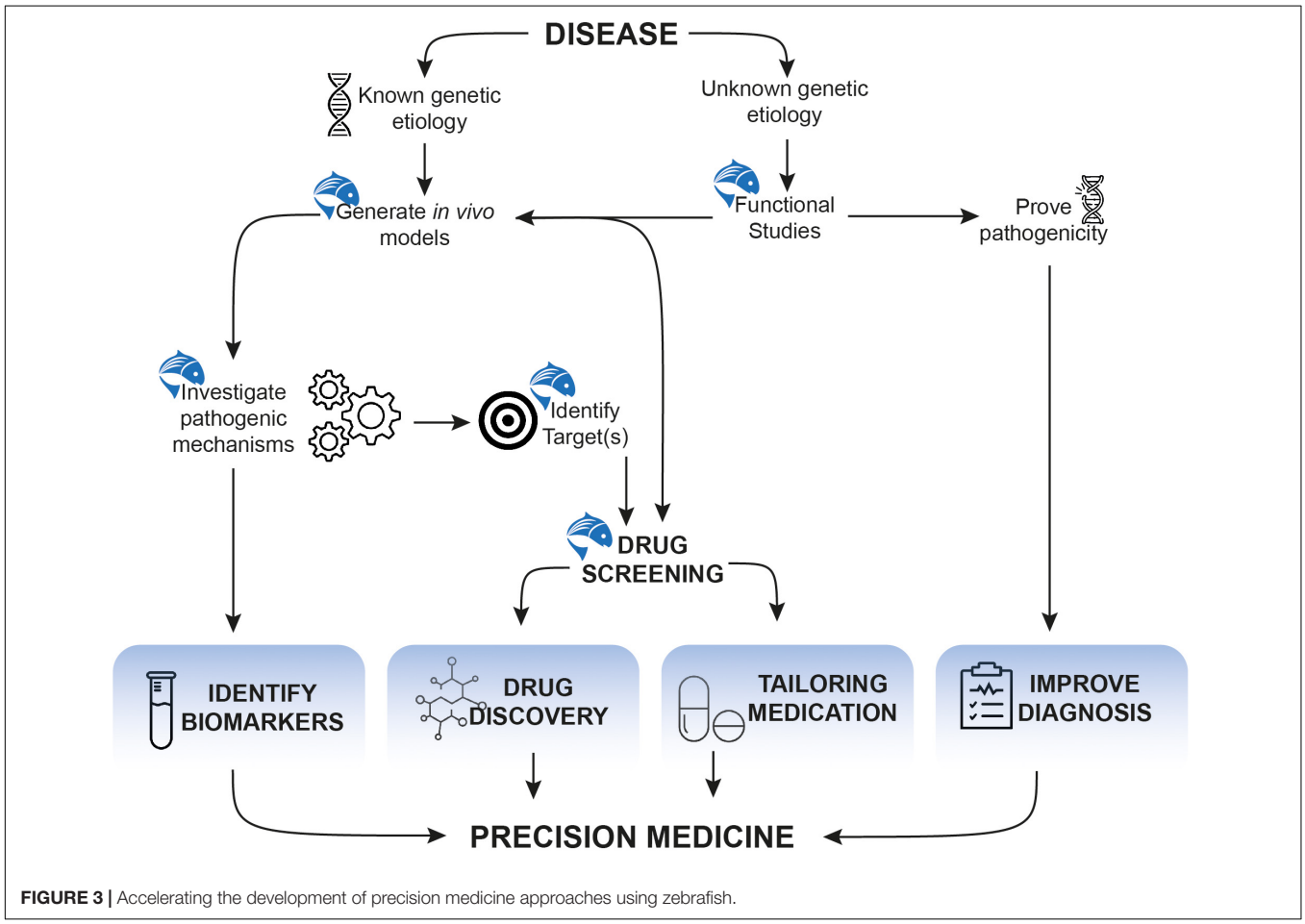

FIGURE 3 | Accelerating the development of precision medicine approaches using zebrafish.

genes involved in the pharmacokinetics and pharmacodynamics of psychiatric drugs (Malhotra et al., 2004; Malhotra et al., 2007; Steimer, 2010; Lisoway et al., 2021). This can bring valuable information for tailoring treatment for anxiety, bipolar disorder, schizophrenia, or ADHD based on the patient's specific genetic profile (Hamilton, 2015; Kose and Cetin, 2018). However, neurology may be lagging in the PGx field behind other specialties such as oncology or immunology include the heterogeneity of disorders and the lack of biomarkers. Moreover, the complex variety of pathogenic mechanisms makes psychiatric disorders particularly challenging to treat with a vague definition and standardization of clinical outcomes among cohorts of patients. That creates an excellent opportunity for researchers to fill this gap with the use of relevant biological models. Despite the advantageous genetic and pharmacological accessibility of zebrafish, it has not been exploited widely in pharmacogenomics. However, its potential in identifying genetic determinants of the physiological response to anesthetic drugs has been recently reviewed by Bedell et al. (2018). Several complex behavioral assays are available to study drug response in zebrafish, including the photo motor startle response (PMR). PMR does not involve any visual organs and is one of the earliest forms of motor behavior in zebrafish (between 30 and 40 h post-fertilization) (Kokel et al., 2010; Kokel et al., 2013). Interestingly, it has been shown that this early behavior is altered in the presence of neuroactive compounds and anesthetics (Copmans et al., 2016; Gauthier and Vijayan, 2018). Therefore, it can be used for high-throughput chemical and/or genetic screens to identify modulators of a variety of drugs, including anesthetics and other neuroactive compounds. Considering the latest progress made in mimicking precise genetic conditions in zebrafish, it is a model well-positioned to investigate the genetic aspects of drug response *in vivo*. This is what Yang et al. (2019) have been proving by testing the sensitivity of zebrafish mutants lacking the expression of specific γ-aminobutyric acid type A (GABAA) receptors subunits to anesthetics. However, the teleost-specific whole-genome duplication that occurred during evolution led to more genes within the zebrafish's genome that can complexify such studies (Sato and Nishida, 2010). Indeed despite the strong genetic similarity between zebrafish and humans, the functional redundancy between paralogues genes can make mimicking patient-specific SNPs challenging and dampen the translatability of pharmacogenetics studies performed in zebrafish to humans.

CONCLUSION

The popularity of zebrafish as a model is well-established as it is a formidable tool in the field of developmental biology, genetics, and pharmacology. Its use fits at multiple impactful levels of the broad precision medicine framework (**Figure 3**). Its success, popularity and utility will continue to grow as novel genetic engineering and innovative screening techniques will continue to emerge.

AUTHOR CONTRIBUTIONS

ÉS designed the figures. All authors wrote and reviewed the manuscript and approved the submitted version.

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Conflict of Interest: ÉS was a co-founder of Modelis Inc. The commercial affiliation did not play any role in this study; in particular, it did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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