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Review

Zebrafish models for the functional genomics of neurogenetic disorders ☆

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ABSTRACT

In this review, we consider recent work using zebrafish to validate and study the functional consequences of mutations of human genes implicated in a broad range of degenerative and developmental disorders of the brain and spinal cord. Also we present technical considerations for those wishing to study their own genes of interest by taking advantage of this easily manipulated and clinically relevant model organism. Zebrafish permit mutational analyses of genetic function (gain or loss of function) and the rapid validation of human variants as pathological mutations. In particular, neural degeneration can be characterized at genetic, cellular, functional, and behavioral levels. Zebrafish have been used to knock down or express mutations in zebrafish homologs of human genes and to directly express human genes bearing mutations related to neurodegenerative disorders such as spinal muscular atrophy, ataxia, hereditary spastic paraplegia, amyotrophic lateral sclerosis (ALS), epilepsy, Huntington's disease, Parkinson's disease, fronto-temporal dementia, and Alzheimer's disease. More recently, we have been using zebrafish to validate mutations of synaptic genes discovered by large-scale genomic approaches in developmental disorders such as autism, schizophrenia, and non-syndromic mental retardation. Advances in zebrafish genetics such as multigenic analyses and chemical genetics now offer a unique potential for disease research. Thus, zebrafish hold much promise for advancing the functional genomics of human diseases, the understanding of the genetics and cell biology of degenerative and developmental disorders, and the discovery of therapeutics. This article is part of a Special Issue entitled Zebrafish Models of Neurological Diseases.

1. Introduction

Zebrafish are used as a model for a wide variety of human diseases, including cancer, cardiovascular disorders, angiogenesis, hemophilia, osteoporosis, diseases of muscle, kidney and liver, and, last but not the least, disorders of the central nervous system. In recent years, zebrafish have been used to study neurodegenerative disorders. Here we review zebrafish models of CNS diseases with an emphasis on studies of degenerative neurological diseases. We also discuss our recent efforts in extending these approaches to psychiatric disorders of development. Although unlikely to yield accurate models for complex psychiatric disorders, genetic insights from studies of zebrafish are pertinent in helping to identify relevant molecular and cellular mechanisms of human pathology and, at this level, promise insight to the development of therapeutics. In particular, the *in vivo* biological validation of variants identified in human genetic and genomic studies provides an important step in defining the pathological nature of mutations.

Apart from being a vertebrate with common organs and tissues such as a brain and spinal cord with conserved organization, the attractiveness of zebrafish as a model lies in its biology and genetics. Zebrafish have large clutches of externally fertilized and transparent eggs, which develop rapidly and in synchrony, with neurogenesis starting around 10 h post-fertilization (hpf), synaptogenesis and the first behaviors around 18 hpf and hatching around 52 hpf. Within 1 day of development, many pertinent features of the CNS appear and can be studied in relatively simple populations of identifiable neurons. For example, the spinal cord is divided into 30 somites each containing fewer than a dozen cell types that form relatively simple circuits. The rapid development of the zebrafish embryo allows for the study of embryonic-lethal mutations as larvae can survive on their yolk for up to a week, allowing studies of gene expression and function throughout early developmental stages.

In addition to its advantageous biology, the second major advantage of the zebrafish as a model is the simplicity and effectiveness of manipulating gene expression for cell biological observations in living embryos with relevance to human pathology. The zebrafish genome is sequenced and, although not completely annotated, over 80% of gene structures are available and show a high degree of synteny (nearest neighboring genes on the chromosomes) across vertebrate species as well as 50–80% homology with most human sequences. Homologs for most human genes can be identified *in silico* and, as described below, can be manipulated by gain- and loss-

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of-function approaches. However, the identification of a human homolog in zebrafish is complicated by the fact that a large number of genes are duplicated. This phenomenon is explained by whole genome duplication that occurred during evolution [1]. The amino acid identity compared to the human gene is always higher for one of the two genes duplicated, indicating that the duplication appeared before the species divergence. Therefore, when a gene is knocked down, it is important to consider that the duplicated gene can compensate for the loss of function of the targeted homolog. Also, the function could be partitioned between the two genes or simply lost for one of the genes. Comparative sequence analysis allows identifying the zebrafish homolog of a human gene. Three major genome browsers, NCBI (www.ncbi.nlm.gov), Ensembl (www.ensembl.org) and UCSC (/genome.ucsc.edu/), can be use to search homologous gene sequences. In many cases, the homologous gene predictions can be found directly from either *HomolGene* (NCBI) or *Orthologue Prediction* (Ensembl). We have found that about half of the hundred or so human synaptic genes on chromosome X (described later) have a predicted fish ortholog, compared to ~30% in *Drosophila* and *Caenorhabditis elegans* using the same databases. If the homologous gene is not annotated or if the transcript prediction is not complete, a BLAST search using nucleotide or protein sequence against the whole genome assemblies (especially scaffolds based on BAC sequencing) can allow the identification of homologous genes. TargetP and SignalP algorithms (www.cbs.dtu.dk/services/) can be used for signal peptide and cleavage site prediction. An additional phylogenetic analysis and multiple alignments of protein sequences (Clustal W) help to confirm the orthology. The syntenic regions can also be compared using *Multi Contig View* (Ensembl). For additional information, the Sanger Institute (www.sanger.ac.uk) has excellent resources about zebrafish sequence analysis. The sequence identity between zebrafish genes and their human homologs is higher for ubiquitously expressed genes than for highly specific genes such as some synaptic genes. The divergence is mainly found in domains important for membrane targeting as signal peptides and often in intracellular domains that contain signaling motifs. Nonetheless, in general, the exon/intron boundaries and regulatory sequences as well as many other types of functional domains are evolutionarily conserved between the species.

As a simple starting point, the zebrafish homologs can be targeted for knockdown by injection of selective anti-sense morpholino oligonucleotides (AMOs) [2]. ZFIN (zfin.org) has the principal database about AMO gene knockdown and phenotype. The parameters to consider in AMO design are the same as that for any oligonucleotide molecule: CG content, stem and loop structure, and the oligo length [3]. Gene-Tools Inc. (see www.gene-tools.com) offers an AMO design service to the researchers purchasing their morpholinos. We recommend always checking if the AMO can bind to off-target sequences by doing a BLAST against NCBI or Ensembl database. AMOs can be designed to target the ATG start site to block translation initiation or they can be used to block splice sites to produce truncated transcripts. The latter have the advantage of sparing maternal transcripts and permit RT-PCR amplification and quantification of the gene knockdown, which is particularly useful when an antibody is unavailable. Further, the splice AMO can be used to mimic splicing defects, nonsense or truncation mutations related to disease. AMOs resist degradation by ribonucleases and are stable for several days, and in our hands when adequately controlled, they usually generate selective phenotypes (discussed below). However, AMOs must be injected intracellularly as they are more of a pharmacological than a genetic tool, although they are more effective in gene knockdown in zebrafish than interfering RNAs. Intracellular injection is feasible in the zebrafish embryo during the first 1–2 hpf as the blastomeres are open to the yolk prior to the 4th cell division. Free-hand injection into the yolk near the blastomere border (Fig. 1) allows for the injection of a hundred eggs or so in 1–2 h, yielding a sufficient number of treated embryos to permit statistical analyses of phenotypes and thus providing a genetic read-out often only 1 day later.

Replacing targeted zebrafish genes with human genes can for many genes be done as simply as by injection of human mRNA simultaneously with the AMO as the latter is designed to specifically target the zebrafish and not the human sequence. We suggest using the zebrafish homologous cDNA if your gene of interest has a highly localized expression pattern like that of synaptic genes. Otherwise, human, mouse, or rat cDNA can be used and easily obtained commercially. NCBI gives the link to cDNA clones for certain genes. The gene can be subcloned in expression vectors like pCS2 [4] or pcGlobin [5] to synthesize the 5′-capped mRNA that will be injected in embryos. The pcGlobin vector has the advantage to have 5′UTR and 3′ UTR from *Xenopus*, and we also note that pcGlobin gives a better yield of mRNA. The cDNA can be tagged to MYC, flag, or HA epitopes to follow the expression in fish. However, large DNA constructs such as plasmids often yield chimeric expression patterns in limited numbers of cells presumably as not all dividing progenitors retain the large constructs. About half of the human genes we have tested permit partial rescue of knockdown phenotypes, which then allows for comparison between wild-type human mRNA and mRNA bearing disease-related mutations. Thus, in the space of a few weeks, each human variant can be validated in knockdown embryos once a clear phenotype is recognized. Finally and as described below, recent transgenic technologies permit the creation of stable, targeted (cell-specific), and inducible lines for better spatially and temporally controlled and more detailed analyses of transgenic function.

2. Degenerative brain disorders

Degenerative disorders of the brain, including Alzheimer's, Parkinson's, and Huntington's diseases, are the first neurogenetic diseases to have been studied using zebrafish [6,7]. Amyloid-beta plaques are the strongest biological marker of Alzheimer's disease and the production of amyloid-beta is regulated by the presenilins 1 and 2 (PS1, PS2). Surprisingly, wt zebrafish zPS1 was found to promote aberrant amyloid-beta42 secretion when expressed in HEK 293 cells, like the abnormal secretion associated with human PS1 mutations, and mutation of one residue in zPS1 abolished this activity [8]. Truncation of zPS1 due to loss of exons 8 and 9 upon injection of splice acceptor AMOs had a dominant-negative effect by increasing PS1 expression [9] and possibly the expression of other Alzheimer's-related genes [10]. Zebrafish zPS2 is expressed at later stages and is regulated by zPS1 [11,12], but its role in amyloidogenesis in zebrafish is unclear. Zebrafish possess two homologues of amyloid precursor protein (APP) with 63–66% homology to their human counterpart [13], and their double knockdown causes a convergence–extension developmental defect that is rescued by human APP mRNA but not by a Swedish mutation related to Alzheimer's disease [14]. The zebrafish APPs possess a functional gamma-secretase complex to produce amyloid-beta [15] and inhibition of gamma-secretase [16] or knockdown of Pen-2 [17], another member of the presinilin complex, blocks Notch signaling to produce a severe neurogenic phenotype. Early studies also examined the tau protein in zebrafish as tau is present in human neurofibrillary tangles, mutations of tau are implicated in dementia and tau may also contribute to Alzheimer's disease. The zebrafish studies have shown that expression of human tau (driven transiently upon injection of a construct with a neural-specific variant of the *GATA2* promoter) results by 2 days in disruption of cytoskeletal structure, tau trafficking, and hyperphosphorylated fibrillar tau staining [18]. More recently, stable transgenic zebrafish expressing mutated P301L human tau and a fluorescent reporter (driven pan-neuronally from the *HuC* promoter) have permitted *in vivo* imaging of defective axonal growth, tau hyperphosphorylation, and the screening of novel therapeutic molecules [19]. These recent developments with zebrafish tau and amyloid open the possibility of further screens for novel therapeutics that, e.g., decrease the hyperphosphorylation of tau

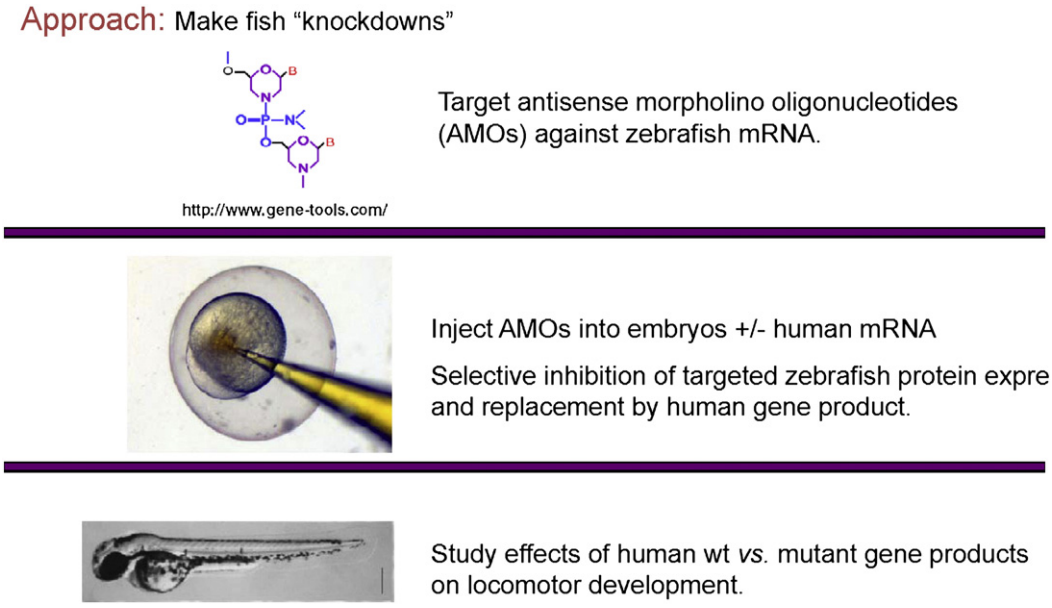

Fig. 1. Rescue of knockdown phenotypes by human mRNA allows for the validation of disease-related variants.

in *HuC* transgenics [19] or amyloid-beta load in amyloid transgenics [20,21].

Research into Parkinson's disease has also used zebrafish as a model [22]. Zebrafish embryos treated with MPTP, a neurodegenerative chemical that reproduces some of the effects of idiopathic Parkinson's disease in mammalian models, demonstrated a loss of dopaminergic neurons, which could be rescued using the monoamine oxidase-B inhibitor deprenyl [23–25]. Half a dozen genes have been identified in Parkinson's disease [26], and several of these have been studied to date in zebrafish. For example, in zebrafish, the mRNA for the ubiquitin processing gene *UCH-L1* was detected by 1 day in the ventral region of the midbrain and hindbrain and in the ventral diencephalon where it was co-expressed with markers of dopaminergic neurons [27]. The zebrafish DJ-1 protein has high (>80%) homology with human and mouse DJ-1 (of unknown function) and is expressed throughout the body [28]. Knockdown of *DJ-1* in the zebrafish did not affect the number of dopaminergic neurons, but zebrafish embryos were more susceptible to oxidative stress and had elevated SOD1 levels, while simultaneous knockdown of *DJ-1* and *p53* caused dopaminergic neuronal loss. Recently zebrafish *Parkin*, another ubiquitin processing gene, was identified (62% homology with human *PARKIN*) and AMO abrogation of its activity leads to a significant decrease in the number of ascending dopaminergic neurons in the posterior tuberculum, which is homologous to the substantia nigra in humans [29]. Screens for compounds promoting (like MPTP) or preventing (such as caffeine) the Parkinsonian phenotype in zebrafish [30] should now be facilitated by the availability of an enhancer trap line expressing GFP from the *vesicular monoamine transporter 2* promoter [31].

In zebrafish, the *huntingtin (Htt)* gene implicated in Huntington's disease has a predicted 70% identity with the human protein [32] and its knockdown disrupts a number of features [33] and causes massive neuronal apoptosis due to reduced BDNF expression by 1 day of development but not earlier [34]. It was recently observed that injecting mRNA coding for the N-terminal fragment of Htt with different length polyQ repeats led to developmental abnormalities and apoptosis in the embryos as early as 1 day later [35]. Embryos expressing a Q102 mRNA [35] or a Q56 plasmid [36] developed inclusions in the cytoplasm. These Huntington's disease models are being used to screen for novel therapeutics that could prevent aggregate formation and clearance, such as anti-prion compounds [35], or embryonic death, such as blockers of autophagy [37].

Another CNS disorder that has been studied recently using zebrafish is epilepsy [38] as clonus-like seizures are induced by convulsant agents [39–42], which has permitted forward mutagenesis screens and the isolation of seizure-resistant mutants [43]. Zebrafish are now being used to screen for compounds that suppress seizures [44]. Furthermore, injection of mRNA with a mutation of the human *PRICKLE1* gene implicated in epilepsy disrupts normal function when overexpressed in zebrafish [45], demonstrating the usefulness of zebrafish for validating mutations of human genes causing brain disorders.

Our group has recently collaborated on the discovery of the MEDNIK syndrome, a rare and severe autosomal recessive neurocutaneous disorder manifested by mental retardation, enteropathy, deafness, neuropathy, ichthyosis, and keratodermia that is often lethal [46,47]. Affected individuals bear an A to G mutation in acceptor splice site of exon 3 of the *AP1S1* gene, which leads to a premature stop codon (Fig. 2A [47]). The *AP1S1* gene encodes the small subunit σ1A of the first (AP-1) of four ubiquitous clathrin adaptor proteins [48–51]. Each one of the adaptor protein complexes is assembled from four subunits, and the σ subunit, the one affected in the MEDNIK syndrome, is part of the AP complex core and is suggested to contribute to its stabilization. Also, together with the μ subunit, the σ subunit is possibly involved in protein cargo selection [50,51]. To demonstrate that the mutation in the human *AP1S1* gene indeed alters the biological function of this gene and underlies the MEDNIK syndrome, we knocked down the function of the homologous *Ap1s1* gene in zebrafish. The zebrafish Ap1s1 protein shares 91% identity with the human protein. To inhibit zebrafish *Ap1s1* mRNA translation, two types of AMOs were designed (Fig. 2A [47]). The first AMO targeted the N-terminal, while the second AMO targeted the acceptor splice site of intron 2 (Fig. 2A), imitating the mutation found in MEDNIK patients. Both AMOs caused similar, severe morphological and behavioral deficits. The 48 hpf KD larva was smaller and had reduced pigmentation compared to the wild-type (WT) larva. Further, blocking *Ap1s1* translation caused disorganized skin formation in general, particularly affecting fin morphology (Fig. 2B; for further information on the epithelial disorders, see Ref. [47]). In addition to the morphological deficits, the 48 hpf KD larva responded abnormally

to touch by coiling the tail instead of swimming away. The compromised touch response of the KD larva could be attributed to a massive reduction (by half) in the spinal neuron population, specifically due to a loss in the interneuron population. The specificity of the AMO effect was further confirmed both by Western blotting and immunolabeling analysis (Fig. 2C and D). Most importantly, we could rescue the knockdown phenotype by co-injecting the AMO with human *AP1S1* wild-type mRNA (Fig. 2B and D), which is not targeted by the *Ap1s1* AMO. In contrast, co-injection of AMO with mutated human *AP1S1* mRNA missing exon 3 (Fig. 2A) failed to rescue the skin and behavioral deficits, suggesting loss of function of this truncated form of protein (Fig. 2C) and confirming the pathogenic nature of the mutation found in MEDNIK patients. Further, co-injection of AMO and an additional human *AP1S1* mRNA isoform found in MEDNIK patients, containing a cryptic splice acceptor site located 9 bp downstream of the start of the 3rd exon, rescued the phenotype (Fig. 2A). The latter result suggests that the predicted in frame protein, lacking only 3 amino acids (Fig. 2C), is functional and may explain the viability of the MEDNIK patients. However, the low expression level of the alternatively spliced RNA (~10%) is insufficient to sustain normal development and function, further emphasizing the importance of AP1S1 in normal development. Our *in vivo* zebrafish study of Ap1s1 function revealed its importance for appropriate neurogenesis and skin development. Accordingly, we could speculate that MEDNIK, a novel neurocutaneous syndrome, is caused by impaired development of various neural networks in the spinal cord and in the brain, explaining the multifunctional human deficits such as ataxia, peripheral neuropathy, and mental retardation, which are concomitant with a perturbation in epithelial cell development in the skin and in the digestive system. We speculate that these generalized effects of *AP1S1*

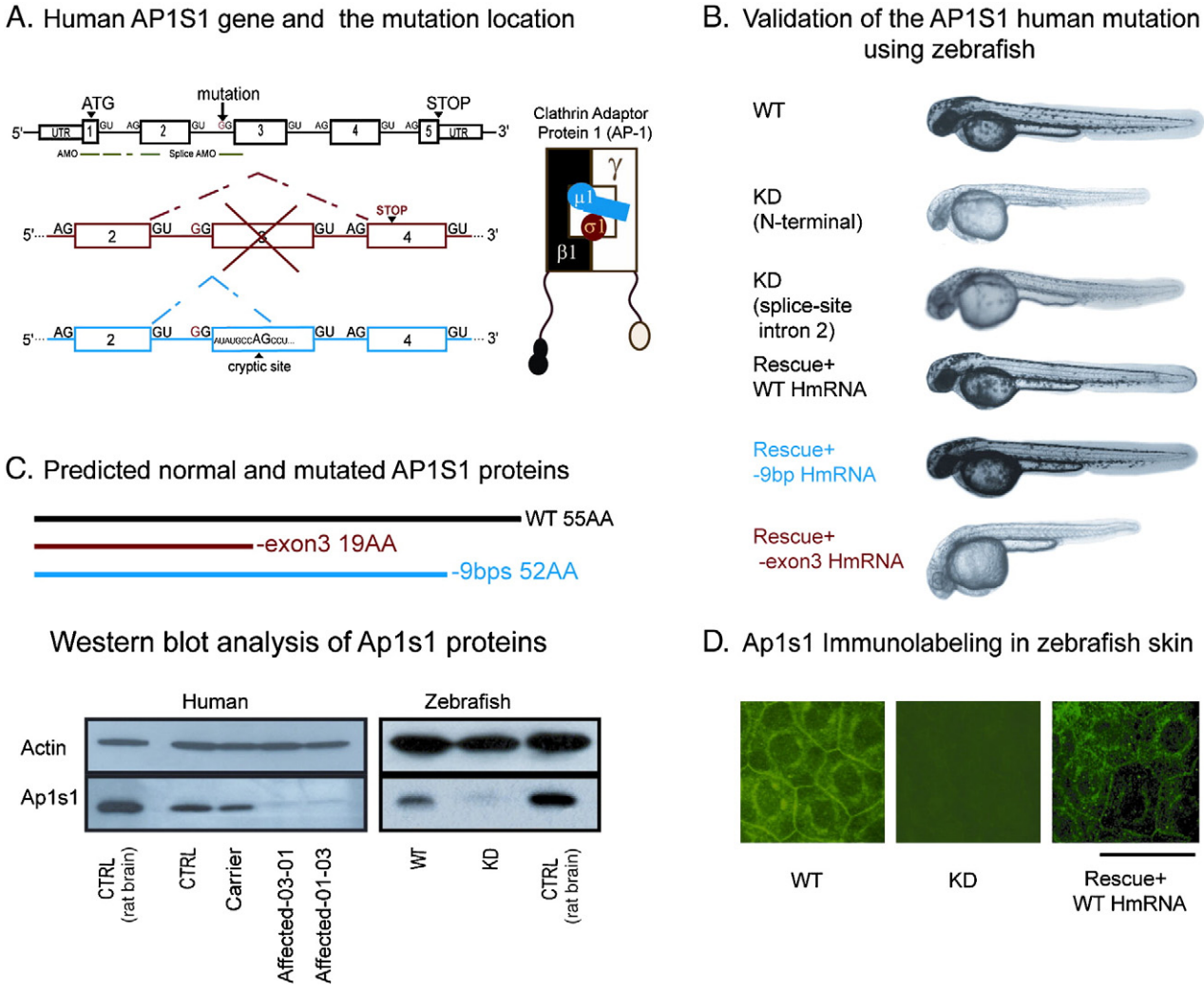

Fig. 2. Evaluating a mutation found in human *AP1S1* gene (MEDNIK syndrome) using zebrafish. (A) An illustration of the human *AP1S1* gene to show the location of the mutation found in MEDNIK patients. An A to G mutation in the acceptor splice site of exon 3 resulted in skipping of this exon and led to a premature stop codon. The use of an alternative cryptic splice site located 9 bp downstream of the start of the 3rd exon resulted in mRNA lacking 9 bp. The location of the two different morpholinos targeting either ATG or exon 3 acceptor splice site of the zebrafish *ap1s1* gene is indicated in green. The inset on the right depicts the clathrin adaptor protein 1 (AP-1) and its 4 subunits. It is the small subunit σ1 (red), which is mutated in MEDNIK patients. (B) Ap1s1 knockdown (KD) in zebrafish using morpholino oligo nucleotides targeting the N-terminal or the splice site (splice site, intron 2) resulted in a similar characteristic morphological phenotype (48 h post-fertilization), which is rescued by co-injection of the wild-type human *AP1S1* (rescue + WT HmRNA) or the mRNA lacking 9 bp (rescue + −9 bp HmRNA), but not by the mutated human mRNA missing exon 3 (rescue + −exon3HmRNA). (C) Illustration of the predicated human AP1S1 proteins: normal protein containing 55AA (WT, black); truncated protein containing 19AA (red), the result of skipping exon 3; and a protein missing 3AA, a result of the alternative splicing (blue). Western blot analysis of Ap1s1 proteins from human (left) and zebrafish (right) to show that Ap1s1 protein is hardly expressed in MEDNIK patients and in ap1s1 knockdown (KD) zebrafish larva. To normalize the Western blot analysis, proteins extracted from WT, KD, and CTRL larvae were incubated with anti-actin. CTRL = control, WT = wild type. (D) Localization of Ap1s1 in skin cells of zebrafish wholemounts is illustrated using anti-Ap1s1 antibody immunofluorescence. Cell membrane (polygonal) and a well-defined perinuclear ring can be nicely observed in both normal (WT) and rescued larvae (rescue + WTHmRNA), whereas only a residual and diffuse staining could be observed in knockdown (KD) larvae (modified from Ref. [47]).

mutation are due to a widespread deficit in vesicular transport and protein sorting.

3. Degenerative spinal cord disorders

Zebrafish have also been proven to be particularly effective as a model for motor dysfunction [52]. Our group has worked extensively on motor neuron diseases, including the most common of these disorders, amyotrophic lateral sclerosis (ALS). The advantage of studying motor neuron diseases in zebrafish consists in the rapid development of the spinal cord and allowing analysis of motor neuron branching patterns as early as 24 hpf. In addition, responses to touch and swimming can be monitored following hatching around 48 hpf [53]. SOD1 (ALS1) is known to cause ALS in 10–20% of familial ALS cases mainly through an autosomal dominant toxic gain of function and has 70% amino acid identity to the zebrafish homologue. Overexpression of mutant SOD1 in zebrafish leads to short motor axons with premature branching [54]. Interestingly, several other genes implicated in motoneuron degeneration show a similar phenotype when tested in zebrafish. Alsin (ALS2) is the gene mutated in juvenile ALS through autosomal recessive mode of action, which leads in most cases to protein truncation. Corresponding to this, in zebrafish embryos, knockdown of Alsin (61% homology) by AMO causes shortening of motor axons and also loss of neurons in the spinal cord [55]. ELP3 was identified through association studies performed in ALS patients and in a *Drosophila* screen for genes important for neuronal survival [56]. Knockdown of this gene (*ELP3*) in zebrafish caused increased branching and shortened motor axons [56]. Recently, TDP-43 was found enriched in inclusion bodies from spinal cord autopsy tissue obtained from ALS patients [57]. Moreover, about 30 mutations have been identified in a considerable number of ALS patients, suggesting that this protein plays an important role in disease pathogenesis [58,59]. The zebrafish z*tardbp* gene product (TDP-43) has 73% homology to the human protein. Knockdown of TDP-43 (*tardbp*) in zebrafish embryos led to motor neuron branching and motility deficits (Fig. 3). This AMO phenotype was rescued by co-expressing human wild-type (WT) TDP-43 (which is not targeted by the AMO) (Fig. 3). However, three ALS-related mutations of human TDP-43 identified in both in SALS and FALS cases (A315T, G348C, A382T) failed to rescue these phenotypes, indicating that these mutants are pathogenic (Fig. 3). A similar phenotype was observed when mutant (but not wt) TDP-43 was overexpressed, with the G348C mutation being the most penetrant (Fig. 3). These results indicate that TDP-43 mutations cause motor defects, suggesting that both a loss as well as a gain of function may be involved in the molecular mechanism of pathogenesis [125]. Finally, it is interesting to note that a number of groups generated ALS2 knockout mice yet failed to observe motor deficits associated with motor neuron degeneration and concluded that ALS2 was not an important gene for ALS [55,60]. However, the first three exons of ALS2 were left intact in these mice. We found that the motor phenotype in zebrafish upon ALS2 KD could be partially rescued upon overexpression of the alternative transcript of the first three exons of ALS2, indicating that complete disruption could in fact be pathogenic in ALS [55]. This nicely illustrates the usefulness of zebrafish knockdown models for more complete disruption of gene function in some circumstances.

Zebrafish have been also widely used to study the functional role of gene mutations in another motor neuron disease, hereditary spastic paraplegia (HSP). Over 30 loci are known for this disorder, and at least 20 genes have been identified to cause spastic paraplegia [61]. Our group identified missense variants in the *KIAA0196* gene at the SPG8 locus and validated these mutations using the zebrafish model (87% homology to the human protein) [62]. Knockdown of the zebrafish homolog of *KIAA0196* gene caused a curly-tail phenotype coupled with shorter motor axons. This phenotype could be partially rescued by the human WT KIAA0196 but not the two mutants identified in HSP patients [62]. Recently, SLC33A1 was identified as the gene responsible for SPG42 [63]. Knockdown of this gene in zebrafish embryos using AMO caused a curly-tail phenotype and defective axon outgrowth from the spinal cord [63]. Finally, knockdown of spastin (SPG4) caused widespread defects in neuronal connectivity and extensive CNS-specific apoptosis [64].

Spinal muscular atrophy (SMA) is an autosomally recessive motor neuron disorder caused by mutations in the survival motor neuron gene (SMN1). In a series of publications, Beattie's laboratory has demonstrated that knockdown of the SMN1 in zebrafish embryos (49% homology to human protein) causes motor axon outgrowth and pathfinding defects in early development [52,65]. Further, in a series of elegant rescue experiments, this group was able to show that a conserved region in exon 7 of the SMN1 gene (QNQKE) is critical for axonal outgrowth and that the plastin 3 (PLS3) may be important for axonal outgrowth since overexpression of plastin 3 rescued the phenotype caused by SMN1 knockdown [66,67]. Finally, transgenic expression of homozygous deletion found in SMA patients that leads to truncated SMN1 protein as well as knocking out the *Smn1* gene in zebrafish caused deficits in the neuromuscular junction formation and death at the larval stage [68].

4. Developmental disorders

Although this developmental model is proving useful in the study of late-onset degenerative diseases, zebrafish are just starting to be used in the study of developmental brain diseases. Interesting speculations have been made on the potential of zebrafish for modeling developmental psychiatric disorders such as autism [69], but the lag in advancing these models is perhaps because the clinical phenotypes are so subtle and specific to humans suffering from disorders such as autism, schizophrenia, non-syndromic mental retardation, and others that zebrafish may appear as a doubtful model. So far (and very recently), the only genes linked to schizophrenia to be studied in zebrafish using AMO methods are *DISC1* and *NRG1* [70]. The receptor tyrosine kinase *Met* has been implicated in cerebellar development and autism, and knockdown of either *Met* alone or both of its *Hgf* ligands (two genes in zebrafish) together using ATG or splice junction AMOs results in abnormal cerebellar and facial motoneuron development [71]. However, neither the human homologs of these genes nor their disease-related mutations have been tested in zebrafish. Our new ongoing genomics approach, based on large-scale re-sequencing of human synaptic genes in patients suffering from some of these disorders, is identifying mutations that can be validated, if not exactly modeled, in zebrafish.

As a part of our “Synapse to Disease” project, we are using a combination of AMO knockdown and gene overexpression to functionally validate novel synaptic gene mutations identified in large cohorts of patients with autism, schizophrenia, or non-syndromic mental retardation. Hundreds of synaptic genes were selected based on published studies and databases. Many genes were chosen because they are X-linked [72] as there are many more affected males than females in autism [73]. Other genes that were selected are those of the glutamate receptor complex because of their association to neurodevelopmental diseases [74]. So far, the exonic regions of over 400 genes have been sequenced, and 15 *de novo* mutations (present in the patients and not their parents) were genetically validated (manuscript submitted). We have identified deleterious rare *de novo* mutations in *Shank3*, *IL1RAPL1*, *NRXN1*, and *KIF17* genes, and all of these genes have orthologs in zebrafish. We have identified a *de novo* mutation in the *Shank3* gene in a patient with autism [75] as Shank3 deletions or duplications were previously identified in patients with autism [76]. Shank3 encodes a scaffolding protein found in excitatory synapses directly opposite to the pre-synaptic active zone. The Shank proteins link ionotropic and

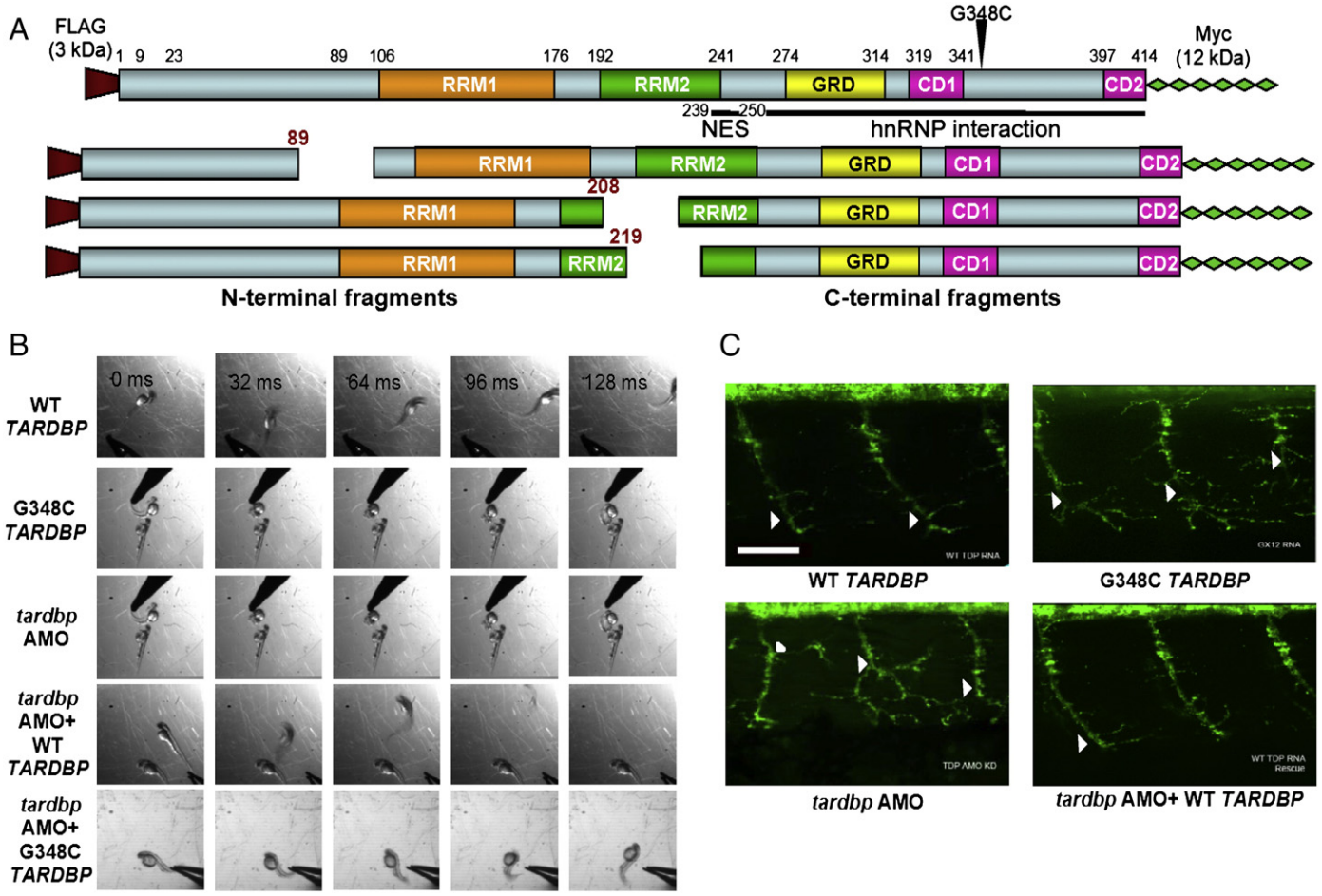

Fig. 3. Motor phenotype of gain and loss of function of TDP-43 in zebrafish. (A) A schematic representation of TDP-43 protein showing the functional domains, localization of FLAG (3 kDa) and myc tags (12 kDa), and sites where the G348C mutation found in ALS patients was introduced by site-directed mutagenesis. (B) Locomotor phenotype. Zebrafish embryos develop a touch-evoked escape response as seen when embryos are injected with WT human TDP-43 RNA. Expression of TDP-43 G348C RNA causes a deficiency in the touch-evoked response. A similar phenotype is observed when the zf tdp-43 expression was knocked down using a specific AMO. This phenotype was rescued with expression of WT but not G348C RNA. (C) Motor axonal deficits. The behavioral (motor) phenotype was selectively associated with a shortening of the axon and premature branching in motor neurons (see G348C TDP-43 and tdp-43AMO) (modified from Kabashi et al., accepted at Hum. Mol. Genet.).

metabotropic glutamate receptor complexes together and to the cytoskeleton to regulate the structural organization of dentritic spines. The splice mutation identified in the *Shank3* gene would result in a truncated protein lacking the Homer, Cortactin, and SAM domains important for spine induction and dentritic targeting [77]. Zebrafish have two Shank3 homologous genes (*shank3a* and *shank3b*). The identity to human protein is overall 65% and 62% for *shank3a* and *shank3b*, respectively, but when we compare every individual functional domain to the human one, the amino acids identity reach 80% and is over 90% for the PDZ domain. The phenotype of Shank3 knockdown zebrafish was not reported in the literature yet, and we observed major motility deficits that could be rescued by the rat mRNA but not by some of the mutations [126].

We also found *de novo* mutations in IL1RAPL1 in autistic patients [78]. IL1RAPL1 and 2 are plasma membrane proteins that belong to a class of the interleukin-1 receptor family characterized by a 150-aa C-terminal domain that interacts with Neuronal Calcium Sensor-1 (NCS-1) [79,80]. A recent study from [81] used *IL1RAPL1*-deficient mouse to show that IL1RAPL1 controls inhibitory network during cerebellar development. In one patient, a *de novo* frameshift mutation in *IL1RAPL1* causes a premature stop of the translation (I367fs) resulting in a truncated protein, lacking the intracellular TIR domain and the C-terminal domain interacting with NCS-1 [78]. Moreover, a large deletion of exons 3 to 7 of IL1RAPL1 in three brothers with autism and/or non-syndromic mental retardation was also identified in our study. Mutations resulting in deletion of the TIR and the C-terminal domains were previously identified in patients with non-syndromic mental retardation [82,83], suggesting that those domains are important to the protein function. Two genes encode IL1RAPL1 in zebrafish (*il1rapl1a* and *il1rapl1b*) and share 69% and 75% identity, respectively, with human protein. The divergence is mainly in the signal peptide (il1rapl1a: 42%, b: 47%). Recently, a study using zebrafish *il1rapl1a* showed that the mRNA is constantly expressed during early embryonic development, indicating that it is maternally provided [84]. In this study, they also showed that knockdown of the other gene, *il1rapl1b*, using an ATG AMO caused a selective loss of synaptic endings in olfactory neurons and that *il1rapl1b* C terminus and TIR domains regulate the synaptic vesicles accumulation and morphological remodeling of axon terminals during synapse formation [84]. These observations suggest that *il1rapl1b* may be essential for synapse formation and indicate how a loss-of-function *IL1RAPL1* mutant could affect brain neurodevelopment and its possible association to autism and non-syndromic mental retardation. To determine more specifically the function of *IL1RAPL1* in the context of schizophrenia, we knocked down the expression of zebrafish *il1rapl1a* with an ATG AMO and observed a severe phenotype with incomplete development of the embryo, which could not be rescued by human mRNA. These preliminary (unpublished) results indicate that *IL1RAPL1* plays an important function during early embryonic development. Further work, such as with splice junction AMOs that

spare maternal transcripts that are essential for early development, is required in order to develop a pathogenic validation.

Neurexins are predominantly pre-synaptic cell-adhesion molecules. They can induce pre-synaptic differentiation by interacting with neuroligins. There are three neurexin genes (*NRXN1*, *2*, and *3*), each of which encodes two major variants (alpha and beta). Only *NRXN1* gene was shown to be disrupted using CNV analysis in autism [85,86]. More recently, *NRXN1* gene has been involved in CNV found in SCZ patients [87,88]. We have identified an insertion of 4 bp predicted to cause a frameshift with a premature stop affecting the two major variants alpha and beta of NRXN1 in SCZ patient. The protein is predicted to miss the transmembrane and intracellular domains. Two orthologs of *NRXN1* have been identified in zebrafish (*Nrxn1a* and *1b*) with an identity to the human protein over 70%. The greatest variability is found in the signal peptide (Nrxn1a, 27%; 1b 32%). An expression analysis showed that all three *Nrxn* genes are expressed during zebrafish embryonic development and some specific isoforms of Nrxn1a expressed at different stage of the development [89]. These results indicate the potential for developing zebrafish models of neurexin mutations. However, the fact that there exist thousands of isoforms of Nrxn1 and the amino acids of the signal peptide are divergent could make the functional validation in zebrafish more challenging.

Kinesin 17 (KIF17) is a member of the kinesin superfamily containing a motor domain for ATP hydrolysis. KIF17 binds to the scafolding Mint1 to transport the NMDA receptor subunit 2B (NR2B) to the dendrites along microtubules [90,91]. In addition, the K^+ channel Kv4.2, a major regulator of dendritic excitability, is also transported to the dentrites by KIF17 [92]. In mice, overexpression of Kif17 enhances spatial and working memory [93]. The expression of KIF17 was shown to be altered in a mouse models for Down syndrome (trisomy 21) leading to learning deficit [94]. We have identified a nonsense mutation resulting in a protein that lacks the tail domain in a SCZ patient. This domain was shown to interact with Mint1 following its phosphorylation by CaMKII [95]. One orthologous sequence was identified in zebrafish with an identity of 61% compare to the human amino acids. The divergence was mainly observed in the motifs important for cargo binding. The zebrafish KIF17 is widely expressed in the nervous system and retina [96]. By using low doses splicing AMO, this group demonstrates that KIF17 is essential for vertebrate photoreceptor development and seems to have little effect on the global zebrafish development. At higher doses of either an ATG or a splice junction AMO that micked the effect of the *de novo* nonsense truncation mutation in schizophrenia, we observed a characteristic dose-dependent morphological trait with stunted, curly embryos [127]. This validates that the nonsense mutation is a loss-of-function pathogenic effect.

Together, these results with several zebrafish developmental genes indicate the potential usefulness of this model for validating brain disease mutations, but clearly each gene requires its own set of carefully designed experiments. Whereas some human genes can be more difficult to study in zebrafish, with others, one can observe faithful disease phenotypes that can be characterized in detail such as for *AP1S1* in MEDNIK and *ALS2* in ALS and thus provide novel insights to these disorders. We have observed some knockdown phenotypes in zebrafish that are not as obvious in knockout mice, which may reflect a lower degree of compensation in zebrafish, perhaps because their reproductive biology is based more on the quantity of offspring rather than necessarily on the quality of developmental compensation.

5. Future directions

Several approaches have the potential of further advancing zebrafish models of human CNS disorders and making them uniquely useful, in particular targeted expression, multigenic analysis and chemical genetics.

5.1. Targeted expression

Most of the approaches taken to date to express foreign genes in zebrafish are based on injection of mRNA for transient expression throughout the embryo. This simple approach is valid for ubiquitously and early expressed genes such as *AP1S1*, *SOD1*, *TARDBP* and members of the ubiquination and beta-secretase complexes discussed above. However, mRNA injection risks expressing genes ectopically, such as in the case of synaptic genes, and is usually only efficient at embryonic stages, prior to mRNA degradation that occurs within a few days. The latter thus limits the time course for phenotypic analysis, which is particularly problematic for studies of neurogeneration in (often) adult-onset human diseases. More specific expression patterns can be achieved by stable transgenesis using the efficient Tol2 transposon system [97], but this is best suited for generating long-term models (rather than for preliminary or large-scale screens) as it is a slower process that requires the raising of founder fish and their out-crossing to verify stable and specific transgenic lines. A number of promoters are available for neural-specific transgenic expression. For example, the *α-tubulin* promoter [98] was the first used to drive pan-neuronal expression and, as described above, the *HuC* promoter expressed in post-mitotic neurons [19] and a neural-specific variant of the *GATA2* promoter [18] are also effective at early stages. More selective subsets of neurons can be targeted using more restrictive promoters such as the dopaminergic-specific *vesicular monoamine transporter 2* promoter [31]. In the spinal cord, which is an important region for studies of motor dysfunction and synaptic transmission, the developmentally conserved transcriptional code [99] has permitted the generation of transgenic lines for selective types of neurons. These include motoneurons: *HB9* [100], *Isl-1* [101], commissural neurons: *Evx1* [102], mostly glycinergic neurons: *Pax2.1* [103]; *vGlyT2* [104], mostly glutamatergic neurons: *Alx/Vsx2* [105], as well as sensory neurons: *Ngn1* [106] and oligodendrocytes: *Nkx2.2a* [107], *olig2* [108] and *sox10* [109].

However, these stable transgenic models also have their limitations, and here, we consider three major ones that can be at least partially avoided depending on necessity: ectopic expression, toxic expression, and genetic background. Although promoter-based stable transgenic lines confer more selective expression patterns, rarely do they perfectly recapitulate the natural expression patterns as often only subsets of enhancer elements are used in generating the transgenic constructs. Indeed, this is a limitation of the lack of a knock-in technology in zebrafish, requiring the integration of novel constructs in a wild-type background (considered below). However, perfectly faithful expression patterns are not always essential as expression in incomplete or expanded subsets of neurons can be useful to study. An approach that provides a more accurate expression pattern is the use of bacterial artificial chromosomes (BACs), such as with the amyloid line mentioned above [21]. In principle (although we are unaware of its practice with zebrafish), humanized models could be generated based on transgenesis with human BACs, as is commonly done with mice. However, vertebrate genes can be hundreds of kilobases in size and are not always completely contained within single BACs. As an alternative, pufferfish (*Takifugu rubripes*) BACs can be used as the intergenic regions are much smaller, an approach we have taken in generating an *Evx1* transgenic line [102], although even in this case less than 80% of the neurons labeled by a selective antibody to evx1 also expressed GFP, indicating that not all evx1 cells transgenically express GFP.

A second issue in considering stable transgenic lines is toxicity, as many of the transgenes bearing disease-related mutations can be embryonic-lethal. A simple approach is to drive expression from a heat shock promoter [110], although this may lose in generalized spatial expression what it gains in restricted temporal activation. A powerful alternative is the use of combinatorial expression systems,

such as the cre-loxP system developed in mice and the yeast Gal4-UAS binary system popular for invertebrate genetics, both of which have found applications in zebrafish [111,112]. Examples are the *Evx1-Gal4* [102] and *HuC-Gal4* [19] lines described earlier. The latter drives transcription in both directions from the UAS in order to express two constructs simultaneoulsy, such as a gene of interest (in this case human P301L tau) and a reporter (dsRed) in the same cells. Another technique for expressing more than one gene from the same construct is to use the self-cleaving viral 2A peptide as a linker between the genes of interest [113].

A final concern is the creation of transgenic lines in a genetic background containing the endogenous gene as (in the absence of knock-in technology) this results in the addition of another gene. One possibility that is rarely used is to create a transgenic in a knockout background. In principle, this can be done if mutants have been isolated in screens for related phenotypes or by TILLING (Targeted Induced Local Lesions in Genomes) [114] available through a new consortium (see https://webapps.fhcrc.org/science/tilling/). A new technology is the targeted lesioning of genes by zinc finger nucleases (ZFN) [115], although this is a complex and expensive technology that at the moment is beyond the reach of small laboratories. Preliminary success in targeting genes in zebrafish is encouraging, and the possibility to use homologous recombination, rather than non-homologous end joining, to repair ZFN-induced double-strand breaks may permit the development of a knock-in approach for zebrafish [116]. Much works remain to be done before this enticing possibility becomes practical, but nonetheless, a number of transgenic approaches are now available for refined studies of gene expression and function in zebrafish, including human genes bearing disease-related mutations.

5.2. Multigenic analysis

Although highly penetrant mutations of single genes are often implicated in CNS disorders, such as the majority considered in this review, most diseases are multigenic in nature, requiring multiple weakly penetrating mutations in predisposing genetic backgrounds in order to produce the disease. Existing genetic models most easily address single gene mutations (or rather have focused on these simple cases), but clearly, the future of these models lies in their ability to provide insights to the nature of genetic interactions in disease. The zebrafish may prove to be particularly amenable to multigenic analysis by a combination of the approaches described above. The use of two or even three AMOs is a simple solution once each AMO is validated. For example, in the context of Parkinson's disease, simultaneous knockdown of *DJ-1* and *p53* was found to cause dopaminergic neuronal loss [28] and knockdown of both *Hgf* ligands was necessary to induce *Met*-related cerebellar defects [71]. Also, photoactivatable caged AMOs can be used to release these at specific time points [117]. This approach seems promising, but unfortunately, caged AMOs are not available commercially yet. Another major limitation of the AMO is the necessity to inject in embryo at 1 or 2 cells stage. An interesting alternative would be to use the vivo-AMOs that allow making gene knockdown and splice modification in adult animals. The vivo-AMOs are modified AMO able to enter cells by endocytosis. They could be conjugated with a dendrimeric octaguanidine (Gene Tools) or a cell-penetrating peptide linked to phosphorodiamidate (PPMO) (AVI BioPharma Inc.). Vivo-AMOs were shown to be effective in mice [118,119], and they are currently tested in adult zebrafish [3].

By performing AMO injections in mutants or transgenics, either stable or inducible, it should in principle be possible to readily test several genes simultaneously or in different combinations, bestowing zebrafish with a polyvalence that is difficult to envisage for other models such as mice. In the case of mutant and transgenic backgrounds, comparative microarray screening [120] becomes feasible, particularly as multiple conditions (e.g., different mutant alleles) permit internal verification and comparison of the array results. A pertinent example is the recent microarray analysis [121] of three alleles of the neurogenic *mindbomb* mutant affecting Notch signaling for which each allele affected the expression of hundreds of genes but less than 100 were commonly affected. Thus, zebrafish have a unique potential for rapidly analyzing multiple genes and dissecting complex genetic pathways.

5.3. Chemical genetics

While zebrafish retain advantages for studying the genetics, biology, and pathobiology of disease genes of interest, they also have the major advantage of being the only vertebrate model amenable to large chemical genetic screens [122]. Small chemical libraries of over 10,000 molecules, including approved drugs and randomly synthesized organic molecules of unknown function, have been screened in zebrafish models of cancer [123] and cardiovascular disorders [124], and some of these are in clinical testing. With the numerous models of brain diseases being developed in zebrafish, this approach holds much potential for drug discovery. This is particularly attractive in zebrafish as functional chemical genetic screens *in vivo* with living embryos offer the potential of discovering therapeutics without prior knowledge or need for rational screening designs. Thus, zebrafish are a unique model ranging from molecular genetic validation to drug discovery with important applications on the horizon for brain diseases, including many complex and untreatable disorders.

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