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Engineering *Xenopus* embryos for phenotypic drug discovery screening ☆

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ABSTRACT

Many rare human inherited diseases remain untreatable despite the fact that the disease causing genes are known and adequate mouse disease models have been developed. In vivo phenotypic drug screening relies on isolating drug candidates by their ability to produce a desired therapeutic phenotype in whole organisms. Embryos of zebrafish and *Xenopus* frogs are abundant, small and free-living. They can be easily arrayed in multi-well dishes and treated with small organic molecules. With the development of novel genome modification tools, such a zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas, it is now possible to efficiently engineer non-mammalian models of inherited human diseases. Here, we will review the rapid progress made in adapting these novel genome editing tools to *Xenopus*. The advantages of *Xenopus* embryos as in vivo models to study human inherited diseases will be presented and their utility for drug discovery screening will be discussed. Being a tetrapod, *Xenopus* complements zebrafish as an indispensable non-mammalian animal model for the study of human disease pathologies and the discovery of novel therapeutics for inherited diseases.

Contents

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1. Introduction

Rare diseases, also referred to as orphan diseases, are classified as diseases that affect a small percentage of the human population [1]. A rare disease has been defined such as one with a prevalence of less than 1 in 2000 and those with 1 in 50,000 are considered ultra-rare [2]. Most rare diseases have a genetic basis, and thus they are present throughout the patient's life. Disease onset may vary and symptoms do not have to appear immediately. Nevertheless, children are particularly affected and about 30% of the affected children will die before reaching their fifth birthday. Rare inherited diseases can vary in prevalence between populations. A disease that is rare in the general population may be common among members of specific ethnic groups. For the European Union, rare inherited diseases are estimated to affect as much as 6–8% of the population, and worldwide the numbers are in the range of 350 million people. Despite the fact that many rare inherited diseases are of life threatening and/or chronically debilitating nature, no or only inadequate treatment options are available. Enzyme replacement for Gaucher's disease, bone marrow transplantation for some forms of leukemia, and gene therapy for rare immune deficiency disorders represent exceptions to this rule [3].

Target-based drug discovery, the standard approach practiced by the pharmaceutical industry for the last 30 years, has mostly failed to address the needs of patients suffering from inherited rare diseases. Phenotypic drug screening has recently been shown to be more efficacious than *target-based* approaches in the discovery of first-in-class small-molecule drugs [4]. Phenotypic drug discovery relies on screening intact cells or whole organisms with chemical libraries of synthetic small organic molecules, natural products or extracts to identify substances that have a therapeutic effect [5–7]. Therefore, phenotypic drug discovery represents a novel, promising approach to meet the therapeutic needs of patients with inherited diseases.

In vivo phenotypic drug screening uses model organisms to identify novel bioactive compounds that could not be recovered with standard in vitro approaches relying on cell culture systems. Seeded on flat culture dishes covered by simplified extracellular matrices and supplemented by artificial culture media, cells used in vitro are no longer in their natural context of the body. If cell lines are used in place of primary cells, they have undergone profound genetic and epigenetic changes in the process leading to immortalization. By contrast, the cells of an intact organism are non-transformed and found in their normal context within organs and tissues, where they are exposed to cell–cell and cell–matrix interactions in a three-dimensional context. Certain drug candidates may require biotransformation in the liver to become active as a metabolite. Such compounds are expected to score negative, if tested in cell culture systems. Drug candidates discovered by their ability to elicit a specific therapeutic effect in an animal model are also likely to fulfill the efficacy and specificity requirements that need to be met by promising therapeutic agents earmarked to enter clinical development. These include proven efficacy, good cell permeability, lack of obvious toxicities, and favorable pharmacodynamic and pharmacokinetic profiles. In vivo phenotypic drug screening, therefore, combines screening and animal testing in one step.

Over the last ten years, embryos of zebrafish (*Danio rerio*) have been very successfully used as whole-organism in vivo bioassay systems to identify novel bioactive compounds with first examples entering clinical testing [8]. Limitations in translating directly from zebrafish to mammalian systems however exist, as demonstrated best by the example of persynthamide, a promising small organic molecule with cell cycle modulating activities. Persynthamide was identified in a screen of a 16,000-compound library for synthetic organic molecules that suppressed the mitotic phenotype observed in the recessive zebrafish cell cycle mutant *crash&burn* (*crb*) [9]. The *crb* mutation affects *mybl2* (formerly known as *bmyb*) gene and causes an increase in the number of mitotic cells in the embryo [10]. Homozygous *crb* mutant zebrafish are viable and thus can be employed for drug screening purposes. Persynthamide was recovered as the only molecule from the chemical library screen able to rescue the mitotic and apoptotic phenotypes observed in homozygous *crb* mutant zebrafish embryos. It was considered a promising antitumor agent because of its ability to suppress the cell cycle defect in *crb* mutant zebrafish without affecting wild-type embryos [9]. The effects of persynthamide on cell cycle regulation were however found to be zebrafish-specific and could not be generalized to

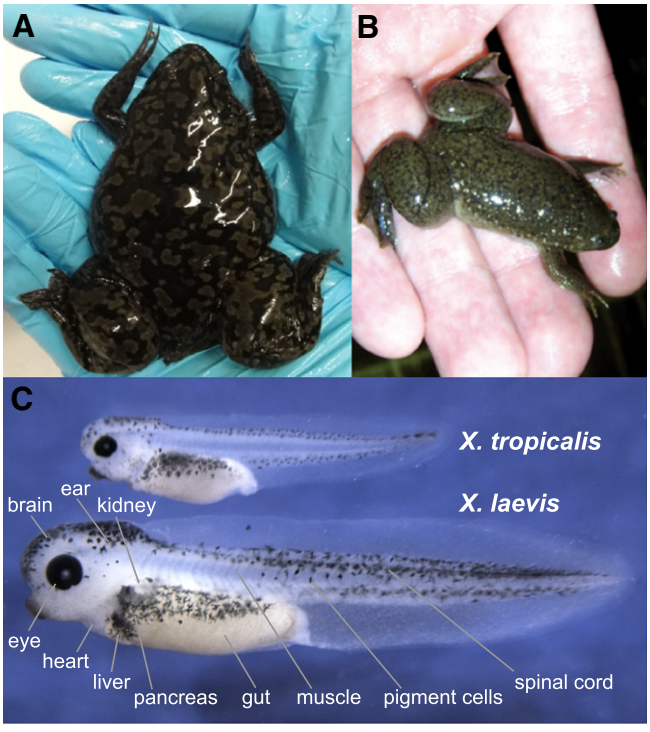

Fig. 1. *Xenopus* frogs and tadpoles. A, B) Comparison of adult female *X. laevis* (A) and *X. tropicalis* (B) frogs. C) *Xenopus* embryos at stage 41 (3 days post fertilization). Positions of the key organs of the vertebrate body plan are indicated. At this stage, they have begun to execute their dedicated physiological functions essential for survival of the embryo.

mammalian cancer cells [8,11]. It is therefore not necessarily given that drug candidates emerging from zebrafish screens will translate directly to mammalian systems. Given the physiological differences and the considerable evolutionary distance between zebrafish and humans, this is not a completely unexpected finding. Some of these problems may be avoided by turning to animal models that are evolutionary closer to humans. Amphibian embryos, in particular those of the African clawed frog *Xenopus* (Fig. 1), have therefore been proposed as an important alternative vertebrate animal model for both the dissection of developmental mechanisms and the discovery of potential therapeutic agents by chemical library screening [12].

Many of the experimental advantages that have made zebrafish a popular vertebrate model for in vivo drug screening purposes also apply to *Xenopus* embryos and tadpoles [6,13]. Embryonic development is rapid and occurs extrauterine in simple salt solutions. The developing larvae and tadpoles become transparent facilitating the detection of tissue and organ defects by visual inspection under a dissection microscope. Importantly, their skin is permeable to small organic molecules. Both animal models harbor the organs and tissues most commonly affected by human diseases, including sensory organs and a central nervous system, a digestive tract, kidneys, a hematopoietic system, a cardiovascular system, and the skin with prominent pigment cells. As tetrapods, *Xenopus* tadpoles will develop limbs and lungs, which are not fish. The closer similarities to mammals are also observed at the level of organ organization and structure, such as the heart, kidneys, lymphatics, and the immune system [6]. For example, *Xenopus* embryos develop a lymphatic vasculature that shares many morphological features with their mammalian counterparts [14]. Similarities in organ morphology also extend to the molecular level. A remarkable conservation in nephron organization between the kidneys of *Xenopus* embryos and adult mice was demonstrated in a large-scale comparative gene expression study [15]. Importantly, tadpoles undergo metamorphosis to mature into land-living adult organisms requiring many physiological adaptations for life on land. As a consequence, amphibian genomes have evolved to accommodate this major change in habitat. The utility of *Xenopus* for drug screening is therefore also underscored by the close homology with the human genome [16]. Finally, *Xenopus* frogs (and amphibians in general) are the only tetrapods to have free-living embryos and thus represent the highest order permitting high-throughput chemical screening in multi-well dishes [6,12,17].

We will focus in the present review on outlining the advantages of using *Xenopus* embryos and tadpoles to develop human disease models and employing these for in vivo drug discovery screening. We will introduce the basic features of the *Xenopus* animal model, discuss genomic resources, and compare methodologies to modify gene functions in *Xenopus* embryos. Importantly, a number of powerful genome editing tools have recently emerged, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the RNA-guided CRISPR/Cas nuclease system, that enable the precise engineering of non-murine model systems for applications in basic science, medicine, and biotechnology. All three genome editing technologies have been successfully adapted to *Xenopus* embryos raising the possibility that for the first time tailor-made *Xenopus* models for human inherited disease can be developed for phenotypic drug discovery purposes. For technical and methodological aspects of performing drug screens in *Xenopus* embryos, the reader is referred to two excellent recent reviews, which appeared elsewhere [6,13].

2. Overview of *Xenopus* as an animal model for biomedical research

2.1. The emergence of Xenopus as the predominant amphibian animal model

Starting in the 19th century, the study of amphibian embryos and tadpoles has led to the discovery of many fundamental mechanisms of vertebrate cell and developmental biology. For example, Johannes Müller, one of the most eminent anatomists of his time, used tadpoles to study excretory organs of vertebrate embryos [18]. Over the next 175 years, research using amphibians led to the discovery and elucidation of embryonic induction, vertebrate axis formation, cell cycle regulation, cloning, and cellular reprogramming [19]. In addition, major contributions were made towards the molecular dissection of the major biochemical signaling pathways (i.e., Wnt, BMP, activin, FGF) and tissue morphogenetic processes (e.g., convergent extension, planar cell polarity). Finally, *Xenopus* oocytes have served for years as an important cellular expression system to study the biochemistry and electrophysiological properties of ion channels, solute carriers, ATP-driven transporters, and signaling receptors. The Nobel Prizes for Physiology or Medicine to Hans Spemann for the discovery of the organizer effect in embryonic development (1935), Tim Hunt for the discovery of key regulators of the cell cycle (2001), and John Gurdon for the discovery that mature cells can be reprogrammed to become pluripotent (2012) testify for the utility amphibians as powerful model systems for biomedical research. In each case, the landmark discoveries were either based entirely on work performed with amphibian animal models or they used them to prove crucial points.

Amphibian embryos and tadpoles became subjects of scientific investigation all the way back to the early 19th century, as demonstrated by Johannes Mueller's description of the excretory organs in tadpoles [18]. Initially, experimental embryologists used the local European and American species, mostly the common frog *Rana* [20]. By the early 20th century, embryos of urodeles were preferred. For example, Ross Harrison used the axolotl *Amblystoma* (American salamander) and Hans Spemann experimented mainly on species of the newt *Triturus* (European newt). Since the 1950s, the African clawed frog, *Xenopus laevis*, has been the most widely used amphibian research organism [20]. In response to injection of human chorionic gonadotropin, which is produced during pregnancy, females can lay eggs year-round. This discovery made by the endocrinologist Lancelot Hogben in the 1930s became the basis of the first reliable pregnancy test for humans and led to the world-wide distribution and use of *X. laevis* in clinical settings [20–22]. Using *X. laevis*, the experimentalists were no longer restricted to the limited breeding seasons of the local amphibians. Several further advantages favored its adoption for research purposes. *X. laevis* are robust frogs that tolerate a wide range of living conditions. Unlike other amphibians, they are obligatory aquatic and thus can be maintained and bred easily in aquaria. They feed on chopped meat and food pellets and do not require living food sources. Since development occurs outside of the mother, each development stage is accessible to experimentation. The embryos are relatively large, which allows the experimentalist to perform microsurgery, tissue transplantations, and other manipulations in ways that are not as easily performed with other vertebrate embryos (e.g. zebrafish, mouse, and rats). Over the years, *X. laevis* has therefore proven to be an ideal laboratory animal for embryological and biochemical studies.

X. laevis is however not an ideal animal model for the purpose of genetics. The genome of *X. laevis* is paleotetraploid most likely due to the hybridization of two parent species (allotetraploidy) around 40 million years ago [23–25]. The duplicated genes resulting from this process are called homeologs (also known as ohnologs or paleologs). Because of functional redundancy, homeologous genes were subsequently silenced and/or lost during evolutionary time in a process known as diploidization or genome streamlining. It is estimated that 50–75% of the duplicated genes have been lost in the genome of *X. laevis* [25]. The remaining gene duplicates in *X. laevis* typically share nucleotide identities in the coding sequences of about 93% [25] and they may retain partially or fully overlapping functions often complicating the generation and study of mutant phenotypes. Importantly, the process of diploidization has returned the ancient paleotetraploid genome of *X. laevis* to a genetically diploid state [26] with a haploid chromosome number of n = 18 [27] (Table 1).

Paleopolyploidy is observed in all extant *Xenopus* species except for *Xenopus* (formerly *Silurana*) *tropicalis*, the western clawed frog, whose genome is a true diploid [28,29]. The genome size and haploid chromosome numbers of *X. tropicalis* (10 chromosomes, 1.7×10^9 bp) are

Table 1
Overview of the characteristics of zebrafish and the *Xenopus* species.

	Danio rerio	*Xenopus tropicalis*	*Xenopus laevis*
Genome			
Evolutionary distance to man[a]	455 million years	360 million years	360 million years
Ploidy	Paleotetraploid	Diploid	Paleotetraploid
Mechanism of polyploidization	Autotetraploidization	n.a.	Allotetraploidization
Haploid chromosome number	25 chromosomes	10 chromosomes	18 chromosomes
Genome size	1.4×10^9 bp	1.7×10^9 bp	3.1×10^9 bp
Protein coding genes[b]	26,206	20,500	25,000–30,000
ESTs[c]	1,488,275	1,271,480	677,911
Total full length ORF clones[d]	11,676	9080	11,525
Practical issues			
Optimal rearing temperature	26–29 °C	24–26 °C	18–22 °C
Eggs per spawning	Up to 200	Up to 3000	Up to 5000
Egg diameter[e]	0.6–0.7 mm	0.7–0.8 mm	1.4–1.5 mm
Larval length at onset of feeding	4 mm	4–5 mm	8–10 mm
Adult body length	4–5 cm	4–5 cm	10–12 cm
Generation time[f]	3–4 months	3–5 months	6–10 months
Life span	2–4 years	>10 years	10–20 years

n.a., not applicable.
[a] References: [40], www.timetree.org.
[b] Gene numbers for zebrafish, *X. tropicalis*, *X. laevis* are based on estimates: [16,25,39].
[c] ESTs, expressed sequence tags; dbEST release 130101 (www.ncbi.nlm.nih.gov/genbank/dbest).
[d] NHI ZGC, *D. rerio* full length cDNA sets (zgc.nic.nih.gov); NHI XGC project, *X. laevis* and *X. tropicalis* full-length cDNA sets (xgc.nci.nih.gov).
[e] Without chorion or jelly coat.
[f] Under optimal husbandry conditions.

approximately half that of *X. laevis* (18 chromosomes, 3.1×10^9 bp) [27,30] (Table 1). *X. tropicalis* retains many of the experimental advantages of its larger relative *X. laevis*, which led in the mid-1990s to its adoption by investigators seeking a genetically tractable amphibian species [19,31,32]. *X. tropicalis* matures in only 4–6 months, is smaller and thus requires less housing space than its larger cousin. Shorter generation times and a small diploid genome have enabled chemical mutagenesis screens and the identification of dozens of mutations affecting different organ systems [33]. In the past years, the first positionally cloned genes have been identified [19]. Despite the unequivocal advantages of *X. tropicalis* for genetic studies, *X. laevis* remains the preferred or exclusively used *Xenopus* species for many experiments (i.e., cell cycle extracts, many developmental techniques, and drug discovery screening). Experimental manipulation and microsurgery is easier due to larger oocytes, eggs, and embryos. For biochemical studies, it is advantageous that the yields of cell extracts per *X. laevis* egg or embryo are about five times higher than with *X. tropicalis*. Finally, the embryos develop at room temperature, which simplifies experimental handling and husbandry.

2.2. Evolutionary distances

When using animal models in biomedical research to gain insights into human biology, developmental mechanisms, and disease pathologies, it is important to be aware of the evolutionary distances to man. The smaller the evolutionary distance, the more reliably the results from studies in model organisms will translate to human biology and patients. This is particularly an issue in drug screening, where the ultimate goal is to develop drugs that are efficacious in human patients. The estimates of the divergence times between *X. laevis* and *X. tropicalis* range from 30 to 90 million years depending on the method used [23,24,34], with the most recent study suggesting a divergence time of about 50 million years [25]. The DNA sequences of *X. laevis* and *X. tropicalis* are 90% identical within the protein-coding regions [35], which is moderately larger in divergence than between mouse and rat (93%) but significantly less than between human and mouse sequences (85%) [36]. Fossil records indicate that the first salamander-like tetrapods (*Acanthostega*, *Ichthyostega*) appear about 365 million years ago in the Late Devonian [37]. On the basis of average evolutionary rates of nuclear genes and a molecular-clock-based method, Lissamphibia, the subclass of Amphibia that includes all living representatives (frogs, salamanders, caecilians), has diverged from the mammalian lineage some 360 million years ago [38].

Beside *Xenopus* frogs, zebrafish has become a popular non-mammalian model organism for the study of embryogenesis, the analysis of gene functions, and the isolation of novel drug candidates [5,6,8]. Similar to *Xenopus*, zebrafish embryos offer the advantages of externally developing embryos, which are virtually transparent and available in large numbers (Table 1). In addition, the zebrafish reference genome sequence was recently released and will accelerate comparative studies with other vertebrate genomes [39]. While zebrafish share many similarities in the basic body plan and at the anatomic level with mammals, they are nevertheless distant relatives. It is believed that Actinopterygii (ray-finned fish), which includes teleosts such as zebrafish, diverged some 455 million years ago from Sarcopterygii (lobed-finned fish, lungfish, and tetrapods) [38,40]. Compared to higher vertebrates, the teleost ancestors of zebrafish underwent an additional whole genome duplication (autotetraploidization) approximately 340 million years ago followed by diploidization [41,42]. Due to this teleost-specific genome duplication, the zebrafish genome is paleotetraploid and possesses 26,206 protein-coding genes, which is more than any previously sequenced vertebrate genome [39]. This has not hampered the isolation of many valuable zebrafish mutations. It is however likely that a pool of redundant genes will remain, whose functions will not be revealed in conventional mutagenesis screens or by standard reverse genetics approaches. Furthermore, the paleotetraploid genome will complicate the generation of zebrafish models of inherited human diseases.

Fig. 2 illustrates the evolutionary distances between zebrafish, the *Xenopus* species and other vertebrate animal models and the paleoploidization events in the lineages giving rise to zebrafish and *X. laevis* are indicated. Overall, the common evolutionary history shared between humans and *Xenopus* is an estimated hundred million years longer than with zebrafish. This has important implications regarding the degree of conservation of genome organization, biochemical mechanisms, and physiological processes between non-mammalian model organisms and humans.

2.3. Life cycle of Xenopus

As many amphibians, frogs of the genus *Xenopus* produce lots, often thousands of eggs, which can be used to make cell-free extracts for

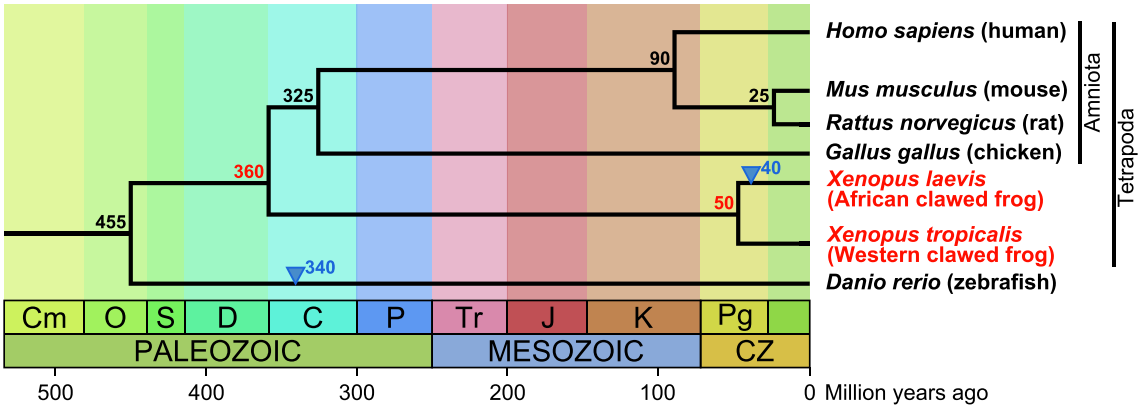

Fig. 2. Phylogenetic relationships, estimated divergence dates and timing of paleotetraploidization events for the principal vertebrate animal models. The branching order of seven model species is shown. Colored horizontal panels represent geologic periods. The divergence times are given in million years ago and represent the mean of time estimates from different studies (see main text for details). Blue arrowheads indicate estimated times of the paleotetraploidization events in the lineages leading to *X. laevis* and zebrafish, respectively. Abbreviations: C, Carboniferous; Cm, Cambrian; CZ, Cenozoic; D, Devonian; J, Jurassic; K, Cretaceous; O, Ordovician; P, Permian; Pg, Paleogene; S, Silurian; Tr, Triassic.

biochemical studies, or they can be fertilized in vitro. Once fertilized, the eggs will develop to tadpoles in simple salt solutions without the requirement of serum or other media supplements. As poikilothermal animals, they have a predictable rate of development, which can be altered by raising or reducing the temperature. This feature greatly facilitates handling of the embryos and experimentation. The adoption of *X. laevis* as the dominant amphibian animal model was further boosted in 1956 by the publication of Nieuwkoop & Faber's Normal Table of *X. laevis* (Daudin) [43]. This substantial and lavishly documented work is an essential resource and sets the standards in the laboratory for the staging of *Xenopus* embryos and tadpoles during development (Fig. 3). Embryonic development is initiated with fertilization, which followed by 12 rapid and synchronous mitotic cycles. The first cleavage can be observed after 90 min and successive cleavage divisions occur every 30 min. During these divisions, the embryo is essentially transcriptionally silent and embryonic development is dependent on maternal factors. Zygotic gene transcription is activated at mid-blastula transition. The cell cycle is desynchronized and lengthens due to the appearance of G1 and G2 phases. Subsequently, the three embryonic germ layers are determined. Embryonic development continues to proceed rapidly: gastrulation, neurulation, and organogenesis, occur within 48–72 h. Unlike zebrafish embryos, *Xenopus* embryos are initially not transparent but they become so as yolk platelets are consumed as development proceeds. Subtle morphological features of the embryo can now be

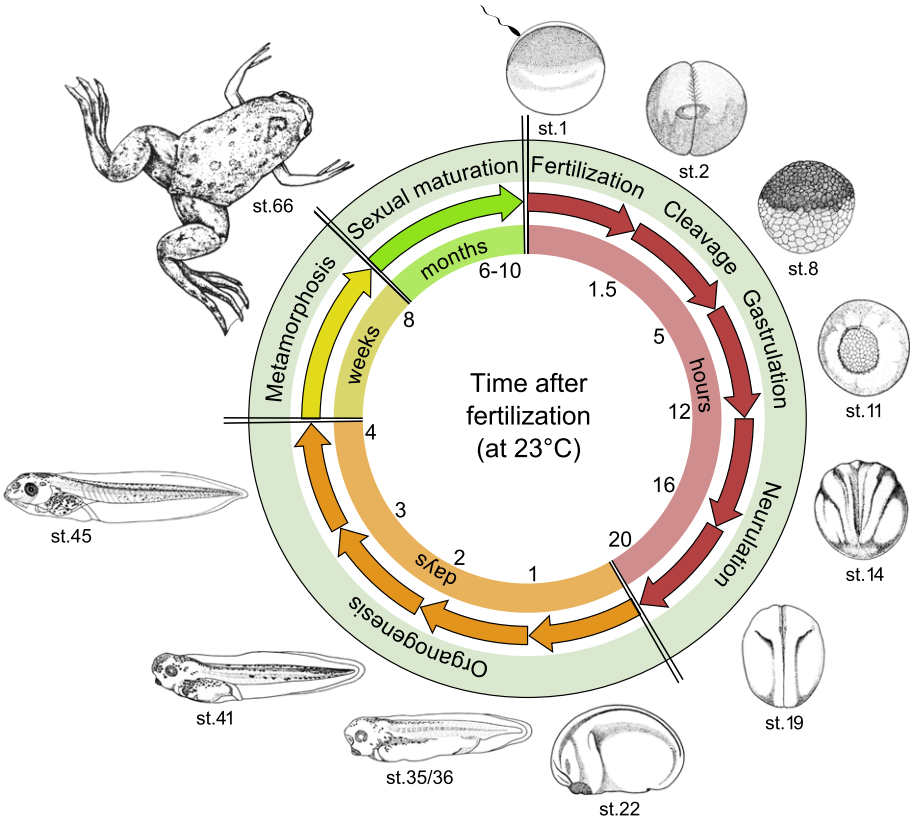

Fig. 3. Timing of the major developmental events in the life cycle of *X. laevis*. Images of embryos and tadpoles were adapted from Nieuwkoop & Faber (1956). For *X. tropicalis*, the rearing temperatures are at 26–28 °C and therefore the generation time is shorter.

recognized. These include discrete organs and prominent structures such as a beating heart, an intricate vascular system, liver, pancreas, kidneys, brain, sensory organs, and pigment cells (Fig. 1C). These organ systems are fully functional as they are critical for the survival of the organism. The free-swimming tadpole will undergo metamorphosis within 2–3 months and develop into an adult frog.

The development of *X. laevis* and *X. tropicalis* appears to be conserved such that the Nieuwkoop & Faber's Normal Tables can be used for both organisms. *X. tropicalis* and *X. laevis* have however different temperature ranges for optimal development with standard lab temperatures of 28 °C versus 22 °C, respectively (Table 1). Due to the higher standard temperature, *X. tropicalis* embryos will develop faster, undergo metamorphosis earlier, and reach sexual maturity earlier than *X. laevis*. The generation times for *X. tropicalis* frogs are therefore about 50% shorter than for *X. laevis*, which puts the species on par with zebrafish (Table 1). Finally, both *Xenopus* species have a long period of fertility, spanning ten years and more. This greatly simplifies the maintenance of transgenic lines and stocks relative to other animal models.

2.4. Animal welfare regulations

Animal experiments carried out within the European Union are since 2010 regulated by EU Directive 2010/63/EU on the protection of animals used for scientific purposes [44]. The Directive applies to experiments using live non-human vertebrate animals, including independently feeding larval forms. Earlier life-stages of animals are not defined as protected. Regarding *Xenopus*, independent feeding is observed after stage 45 (4–5 days post fertilization) [43]. At this timepoint, most experimental procedures are terminated and thus they are not subject to regulation by the Directive. This fact is particularly important for drug screening and toxicology testing procedures using live *Xenopus* embryos. For several reasons, they are usually terminated before the onset of feeding. First, the addition of food to the media can potentially interfere with the activity of the test compounds. Second, older embryos will require larger volumes of test media and the assay will consume more of the precious test compounds. Thirdly, tadpoles will become too large to be kept in 96- or 48-well dishes. Finally, antisense morpholino oligonucleotides (MOs) used to disrupt gene functions in *Xenopus* embryos lose their effectiveness once embryos are a few days old. On the basis of comparative transcriptome analysis, *Xenopus* embryos at stage 43 correspond best to mouse embryos at embryonic day 18.5 [45]. The experimental window until *Xenopus* embryos reach stage 45 therefore covers a period roughly equivalent to the entire gestational period of the mouse embryo. Overall, *Xenopus* stage 45 is therefore a convenient endpoint for many experimental purposes. This fact significantly simplifies the licensing procedures required to perform experiments using the *Xenopus* animal model.

3. Genomics resources for *Xenopus*

The long-term viability and acceptance of *Xenopus* as an important vertebrate model organism for biomedical research will strongly depend on the development of state of the art genomic resources. This has been recognized by the *Xenopus* community [46] and has in the past ten years led with the support of the US National Institutes of Health (NIH), the Joint Genome Institute (JGI), the Wellcome Trust in the UK, and the National Institute of Genetics in Japan to the unprecedented development of genomic resources for both *Xenopus* species.

3.1. Genome sequences

Since the beginning of the new millennium, the genomics revolution has tremendously transformed the use of animal models in biomedical research and led to the development of novel, highly sensitive tools to profile in a genome-wide manner changes in the transcriptome and proteome of cells, tissues, organs and even either organisms. High-quality, well-annotated genome sequences form the basis of this revolution and they are the most important resource of any model organism community. Genome assemblies are essential for comparative genomics, gene discovery purposes, the identification of candidate genes in positional cloning projects, and the study of gene organization and evolution.

In 2010, the diploid genome sequence of *X. tropicalis* (v4.1) was published [16]. Strikingly, the *X. tropicalis* genome shows high conservation with mammalian genomes. For example, it is highly syntenic with the human genome, with regions of synteny frequently spanning more than a hundred genes. Nucleotide sequencing of the *X. laevis* genome has proceeded at a slower pace, partly due to its paleotetraploid genome, but in 2012 a first draft was released on Xenbase, the *Xenopus* model organism database (http://xenbase.org) [47]. In December 2013, the International *Xenopus* Genome Consortium, which includes research laboratories from the US, Japan, France and the UK, have made the newest *X. laevis* (v7.1) and *X. tropicalis* (v8.1) genome assemblies available to the public through Xenbase. The *X. laevis* (v7.1) genome assembly has improved contiguity with 2.7 Gb total scaffold length and 50% of the assembly in contigs >19.3 kb (contig N50). In addition, the Japanese National Institute of Genetics has produced a draft genome assembly of the *X. laevis* J strain (XenVis2.0) with contig N50 >10.9 kb (http://xenopus.lab.nig.ac.jp). The *X. tropicalis* genome (v8.1) was generated using improved computational methods incorporating additional bacterial artificial chromosome (BAC)-end data and by integrating a dense genetic map resulting in a more contiguous assembly mapped to chromosomes with 50% of the contigs >72 kb in length. Genomes of human, mouse, *Drosophila*, and *Caenorhabditis elegans* have become highly refined over the last decade. By contrast, the *Xenopus* genome assemblies are still relatively incomplete harboring gaps in the assembled sequences, which has the potential to disrupt the gene modeling process [48]. It is likely that these problems will be resolved in the near future as the quality of the *Xenopus* genome assemblies is rapidly improving with the plummeting costs of DNA sequencing.

The current genome assemblies represent already now invaluable resources for the *Xenopus* community. For example, the identification of *Xenopus* orthologs of human disease genes no longer depends solely on homologies scores derived from amino acid sequence comparisons, but will also draw on examining the genomic context, i.e. conservation of synteny with the human genome. Given the paleotetraploid origin of the *X. laevis* genome, it is now possible to determine unequivocally whether a given human gene is represented by one or two orthologs in the *X. laevis* genome. This information is especially important, when planning experiments to disrupt gene functions in *X. laevis* as it may reveal potential genetic redundancies. Furthermore, the *Xenopus* genome assemblies enable a high degree of accuracy when designing gene modification tools, such as MOs, TALENs, and CRISPR/Cas (see below).

How do the *Xenopus* genomes compare to zebrafish? The zebrafish genome possesses an estimated 26,206 protein coding genes, which is more than any previously sequenced vertebrate genome and it has a higher number of species-specific genes than human or mouse [39]. The increased gene number is likely to be a consequence of the teleost-specific genome duplication. By contrast, the *X. tropicalis* genome is estimated to contain about 20,500 protein-coding genes on the basis of homology-based gene prediction models and *Xenopus* nucleotide sequence resources [16]. This is very much in line with other diploid mammalian genomes, such as mouse (20,210 genes) and humans (20,687 genes) [49,50]. For the paleotetraploid *X. laevis* genome, it is estimated that at least 25–50% of the duplicated homeologs have been retained [25]. This will amount to a gene number ranging between 25,000 and 30,000 genes. It is therefore fair to assume that the gene numbers of *X. laevis* will be comparable to those for zebrafish (Table 1). Given that the polyploidization event in the lineage leading to *X. laevis* occurred much more recently than the teleost-specific genome duplication (40 vs. 340 million years ago, see also Fig. 2), the process of neo- and subfunctionalization of homeologs is likely to be much less advanced in *X. laevis* than in zebrafish. With the rapid

improvements of the *Xenopus* genome assemblies, better gene annotations and more accurate estimates of the gene numbers for both species should become available soon.

3.2. ESTs and full-length cDNAs

Expressed sequence tags have been instrumental in gene discovery and gene sequence determination [51]. Each expressed sequence tag (EST) is a short single-read transcript sequence of a cDNA sequence and thus represents a portion of an expressed gene. Since the initiation of systematic EST sequencing in 1991, more than 8 million human ESTs have been deposited in public EST database of GenBank (dbEST release, 1 January 2013). Due to large-scale EST projects, the two *Xenopus* species are represented by nearly 2 million ESTs drawn from cDNA libraries of different embryonic stages and from several adult organs (Table 1). This rich resource complements the *Xenopus* genomes and serves as an independent tool to validate the genome assemblies and refine the predicted gene models. Furthermore, the analysis of EST databases permits studies of alternative splicing and the identification of developmental stage- and tissue-specific genes [52]. Finally, EST collections are also useful nucleotide sequence resources to estimate possible single nucleotide polymorphisms (SNPs), when designing tools for targeted gene knockdowns or genome editing (see below).

The cDNAs used for EST generation are typically individual clones from cDNA libraries and many contain the complete open reading frame (ORF). On this basis, the *Xenopus* Gene Collection (XGC) project of the National Institutes of Health (NIH) selected EST and generated full-length ORF sequences for *X. tropicalis* and *X. laevis* (http://xgc.nic.nih.gov), which are available for public use [46]. At the time of conclusion of the XGC project in 2008, it had generated 11,515 full-length ORF sequences for *X. laevis* and 9080 for *X. tropicalis*. The cDNA clones were cloned into eukaryotic expression vectors carrying promoters for in vitro transcription and can be purchased from distributors of the IMAGE consortium. Besides providing access to mRNAs of many *Xenopus* genes for overexpression and gain-of-function studies, arrayed collections of full-length cDNAs have enabled a more sensitive and efficient approach to expression cloning than was previously possible with random cDNA pools by reducing redundancy [53,54]. The cDNAs of the XGC project represent however less than 50% of the genes anticipated from the *Xenopus* genome projects. The *Xenopus* ORFeome project was therefore launched in 2011 with the aim of developing cDNA libraries containing all non-redundant ORFs of both the *X. laevis* and *X. tropicalis* genomes [55]. With the anticipated completion in 2016, the *Xenopus* ORFeome is likely to become an important resource for a variety of applications, including high-throughput expression cloning and proteomic screening.

3.3. Xenbase, the Xenopus database

Organism-specific databases are essential tools for all major animal models in biomedical research. Xenbase (http://www.xenbase.org), the *Xenopus* model organism database, is a crucial resource for the *Xenopus* community [47,56,57]. It contains information about all aspects of research involving the two primary *Xenopus* species, *X. tropicalis* and *X. laevis*. This includes access to the genome assemblies, gene annotation, epigenomic data, gene expression resources, plasmid and antibody reagents, experimental protocols, references, and community information. Most importantly, Xenbase features its own genome browsers and provides access to the most current versions of the *X. tropicalis* and *X. laevis* genome assemblies. It also coordinates the generation and validation of *Xenopus* gene models and annotations. Xenbase has created specific web pages for more than 16,000 annotated genes, which are updated on a regular basis and linked to several other databases, such as NCBI or UniProt. Furthermore, gene expression data are collected from the *Xenopus* community and by mining the *Xenopus* literature. Finally, Xenbase acts also as an interface for the *Xenopus* stock centers in US and Europe providing information on the availability of transgenic *Xenopus* lines and research reagents. The aim of Xenbase is to incorporate all of these different types of data sets to promote and facilitate the use of *Xenopus* as a versatile non-mammalian animal model to study processes relevant to human health and diseases.

4. Modification of gene functions in *Xenopus*

The ability to introduce gene modifications in vertebrate model organisms is an essential step in the elucidation of novel gene functions. Reverse genetics seeks to alter a gene function and document the

Table 2
Comparison between current gene manipulation tools for *Xenopus*.

Technique	Advantages	Disadvantages
CRISPR/Cas	– Site-specific mutation – Easy to produce guide RNAs – High efficiency – Off-target effects rare in vivo – Heritable traits	– Some limitations in the gene targeting range – Genotyping is required
ENU mutagenesis/TILLING	– Recovery of specific point mutation in gene of interest possible – Heritable traits	– Random mutagenesis process – Low efficiency – Time consuming – Labor intensive – Considerable animal space requirements
Morpholinos	– Versatile loss-of-function approach – Technically simple – Rapid implementation – Generation of large amounts of homozygous mutants possible – No genotyping required – Targeting of multiple gene products possible	– Off-target effects possible – Transient knockdown limited to embryos and tadpoles – Incomplete gene silencing possible – Non-heritable modifications – Testing of knockdown efficiency is required
siRNAs		– Ineffective in zebrafish and *Xenopus* embryos
TALENs	– Site-directed mutagenesis – Unlimited gene targeting range – High DNA cutting efficiency – Facilitated homologous recombination – Heritable traits	– Manufacture more complex than CRISPR/Cas – Off-target effects possible – Genotyping is required
ZFNs	– Site-directed mutagenesis – Facilitated homologous recombination – Heritable traits	– Limitations in the gene targeting range – Low DNA cutting efficiency – Difficult to manufacture – Off-target effects and toxicity – Genotyping required

phenotype arising from this manipulation. Reverse genetics also enables the generation of animal models for human inherited diseases, which can be employed for the study of disease pathologies and may be used for the purpose of drug discovery and development. In the mouse, sophisticated gene modification approaches using homologous recombination strategies have been developed to modify genes by targeting the genome of embryonic stem (ES) cells, which are then used to reconstitute tailor-made mouse mutants [58]. However, considerable challenges still remain. Generally, the efficiency at which engineered constructs are correctly inserted into the chromosomal target sites is low. Furthermore, the screening and selection of correctly modified ES clones is time-consuming and labor-intensive. Finally, despite lots of efforts, the establishment of reliable ES cell lines or their equivalents has to date failed for all other vertebrate model organisms. Alternative methods to interfere with gene functions in non-murine model organisms have evolved from the use of function-blocking antibodies and dominant-inhibitory proteins and to the various forms of antisense oligonucleotides, siRNA, and small molecule inhibitors of enzymes or signaling components. While these methods can be very effective under specific conditions, the inhibition of gene product activity is typically transient and not heritable. Random mutagenesis screens have been employed to recover mutations in specific genes, but there is no guarantee that the resulting mutation will be a null. Recently, many of these problems have been resolved with the introduction of new classes of powerful gene editing tools [59], which for the first time permit the induction of targeted mutations in non-murine model organisms. We will outline the strength and weaknesses of the most important reverse genetics methodologies for *Xenopus* (Table 2).

4.1. Random mutagenesis and TILLING

Forward genetic approaches, where random mutagenesis is used to introduce gene mutations and mutant individuals are selected on the basis of aberrant phenotypes, have been successfully used to generate mutants in the diploid *X. tropicalis* [60]. Insertional mutagenesis using transposons has been an attractive strategy, as the cloning of the disrupted loci is simple [61–63], but the gene disruption efficiency remains unclear. Alternatively, chemical mutagenesis screens have used *N*-nitroso-*N*-ethylurea (ENU), an alkylating agent, to generate point mutations at high rates. In a first ENU screen, dozens of mutations affecting different organ systems have been recovered [33]. Of particular interest are mutations affecting limb and lung development as their recovery requires a tetrapod genetic system such *X. tropicalis* and thus cannot studied in fish model systems. Furthermore, limb regeneration in amphibians, which serves as an important paradigm in regenerative medicine, can now be dissected genetically using *X. tropicalis*. The release of the *X. tropicalis* genome assembly [16] and the genetic map [64] has greatly simplified the identification of mutations. Subsequently, the first positionally cloned genes have been identified in the past years [65–67]. The collections of chemically-induced *X. tropicalis* mutants are also a valuable resource for the identification of animals carrying mutations in genes of interest by TILLING (targeting induced local lesions in genomes), a sensitive high-throughput screening method [60]. Initially, TILLING was developed using non-sequencing based methods [33], but high-throughput screens have been hampered by PCR amplification steps, which tend to introduce bias and errors. These problems have however been recently overcome by next generation sequencing of a subset of specific genes of interest or high-throughput exome sequencing [60]. While exome sequencing is identifying a growing number of *X. tropicalis* mutations, the TILLING methodology is labor-intensive and the recovery of specific gene variants, e.g. those mimicking human disease gene mutations, cannot be guaranteed.

4.2. Morpholinos (MOs)

The use of small interfering RNA (siRNA) as a rapid method for knocking down gene expression in mammalian cell cultures is well established [68] but is ineffective in zebrafish and *Xenopus* embryos due to the lack of endogenous Argonaute endonuclease activity [69]. Given these limitations, injection of antisense morpholino oligonucleotides (MO) has become the most popular way to disrupt a gene function in zebrafish and *Xenopus* embryos [70–72]. MOs are nucleic acids characterized by morpholine rings, which replace the ribose or deoxyribose moieties, and linked together by non-ionic phosphorodiamidate groups [73]. They have become the antisense oligonucleotide chemistry of choice for gene knockdowns in non-mammalian embryos due to their efficiency and specificity, resistance to nucleases, good solubility in aqueous solutions, low toxicity, and low production costs. The mechanism of action of MOs relies on the stability of the duplex formed on the target RNA that leads to steric hindrance. Two main strategies are used to specifically alter gene functions using MOs: inhibition of translation, and modification of RNA processing (Fig. 4). In addition, specific MOs can also be used to block maturation of microRNA (miRNA) [74]. As the design of MOs does not require genomic sequence information, MO-mediated translational inhibition is the most commonly used strategy. The antisense MOs are most effective in blocking translation when

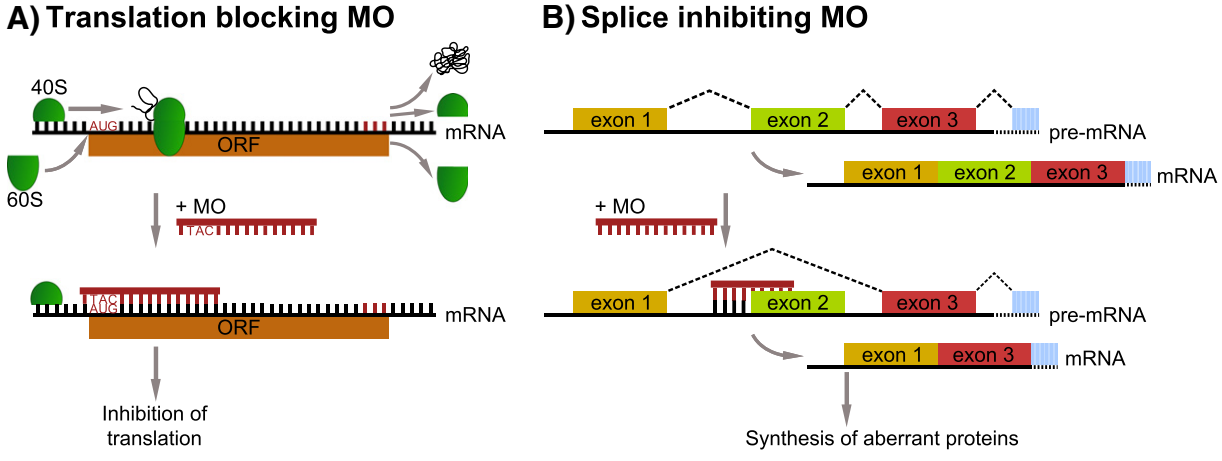

Fig. 4. Designing morpholinos (MO) for gene knockdowns. A) Translation blocking. Hybridization of the MO to the region of the initiation codon (AUG) or the 5′-UTR blocks scanning by the small ribosomal subunit. The large ribosomal subunit is therefore not recruited at the translational start site and no functional ribosome is assembled. Consequently, protein synthesis is disrupted. B) Splice inhibition. The MO is designed to hybridize to an intron–exon junction of the pre-mRNA. As a result, this junction and the corresponding exon (*green*) are skipped. Exon skipping will introduce a deletion and possibly a reading frame shift leading to the synthesis of a truncated and aberrant protein.

they are directed to the 5′-untranslated region (5′-UTR) or the region spanning the AUG start codon of the mRNA [75] (Fig. 4A). Inhibition of translation is achieved by interfering with the ability of the small ribosomal subunit to scan through the 5′-UTR to the start codon. Consequently, no protein product is synthesized. When MOs are targeted against the protein coding sequence, even if this is only a few bases 3′ to the AUG translational start site, they have essentially no effect [75]. It is believed that the ineffectiveness of MOs against down-stream coding sequences results from the ATP-driven unwindase activity of fully-assembled ribosomes, which is effective in displacing MOs from the mRNA [76]. Given this particular mechanism of action, the off-target effects of MOs are strongly reduced, but not entirely eliminated. The effectiveness of the MO knockdowns can be evaluated by measuring the levels of the targeted gene product by immunostaining or immunoblotting. In the absence of a specific antibody, an epitope-tagged test construct can be used as an alternative [77].

MOs are small enough to freely diffuse into the nucleus, where they can hybridize to pre-mRNA transcripts that have not yet undergone splicing. This fact is exploited in an alternative strategy, where splice-inhibiting MOs are designed to interfere with RNA maturation by targeting exon–intron junctions (Fig. 4B). This may result in exclusion of the exon (exon skipping), retention of an intron, or activation of cryptic splice donor or acceptor sites. As a consequence of modifying the splicing patterns, a reading frame shift may be introduced leading to a premature stop codon. Therefore, splice-inhibiting MOs, if correctly designed, can be used to reduce expression of a given gene. The efficiency of splice-inhibiting MOs can be examined by reverse transcription (RT)-PCR, cloning and sequencing of the wrongly spliced mRNA species. No specific antibodies are therefore required. However, a mixture of different splice products are frequently obtained, which may reduce the effectiveness of splice-inhibiting MOs [77]. Furthermore, splice-inhibiting MOs are ineffective at blocking maternal mRNA.

Irrespective of the MO knockdown strategy used, system-wide delivery of MOs is easily achieved by microinjection into fertilized eggs, but the inhibitory effects of MOs only last into the swimming tadpole stages as they become gradually diluted. In many cases, this limitation may not pose a serious problem as the knockdown experiments are terminated prior to the onset of feeding (see 2.4). Caged MOs or photo-MOs can be used for conditional gene knockdowns in a specific temporal context or a defined tissue or organ [78–80]. These MOs are either activated or inactivated under UV light and allow for finer spatiotemporal control, including knockdowns in older animals. General toxicity and off-target effects of specific MOs can also be problematic. In zebrafish, a significant number of MOs can also trigger neural apoptosis mediated by a p53-dependent pathway [81]. Interestingly, a similar MO-induced p53-dependent cell death pathway has to date not been observed in *Xenopus* embryos. In some cases, complete depletion of a gene product using MOs can turn out to be difficult or impossible to achieve, which makes it difficult to obtain reproducible phenotypes. These problems can however be overcome by designing and testing several MOs targeting the same gene product. Furthermore, control MOs, such as MOs harboring several mismatches, should be used to assess for specificity. Where it is possible, the knockdown phenotypes should be rescued by the coinjection of target mRNA engineered with silent mutations that render it resistant to MO knockdown [70].

The abovementioned limitations of MOs have however to be balanced against the considerable means it takes to generate targeted gene mutations and the time – months to years – required to generate homozygous mutant animal lines. Importantly, the phenotypes of genetic mutants carrying recessive mutations will only manifest in a quarter of the progeny of heterozygous mutant parents. By contrast, thousands of homozygous mutant *Xenopus* embryos, where 80–100% of the animals display a fully penetrant phenotype, can be generated in a single day by microinjection of optimized MOs. There is also no requirement for confirmation by genotyping. MOs also significantly facilitate the study of maternal mRNAs. Maternal mRNAs (and proteins) are encoded by the mother's genome and are supplied to the developing oocytes. Since zygotic gene transcription is initially inactive in early embryos, these maternal factors frequently fulfill important functions [82,83]. Hence, for these cases, the genotype of the mother determines the phenotype of the embryo. The function of maternal mRNAs (but not proteins) can be easily studied by injecting translation-blocking MOs into the fertilized egg. By contrast, genetic approaches to determine maternal mRNA functions are more complicated and time consuming as they involve the breading of homozygous mutant mothers and the examination of the phenotypes in their offspring. For these reasons, MOs are presently still considered the most popular method to disrupt gene functions in *Xenopus* embryos.

4.3. Zinc-finger nucleases (ZFNs)

While injection of MOs can be very effective at knocking down specific gene functions in embryos, there is no alteration of genetic information and hence no transmission of the modifications to the next generation. Methods for gene inactivation at the genomic level have therefore been long sought after to overcome the limitation of antisense MO knockdowns. Furthermore, there is an urgent need for methods to introduce site-specific mutations into a gene of choice in non-murine model organisms. Artificial site-specific nucleases such as zinc-finger nucleases (ZFNs) and transcription activator-like (TAL) effector nucleases have emerged as powerful technologies for genome editing by enabling gene disruption and gene replacements [59,84]. These engineered DNA scissors induce in vivo double strand breaks (DSBs) at a desired site in the genome and activate repair processes that can be harnessed for genetic modification purposes.

ZFNs have been developed as the first class of targeted nucleases [85,86]. They are engineered by combining a series of Cys_2His_2 zinc-finger DNA binding domains with *FokI* restriction endonuclease catalytic domain [87]. Zinc-finger DNA binding domains primarily bind 3 base pairs per zinc finger and the binding-site depend upon key residues within the 30-aa zinc-finger. Typically, arrays of 3–6 zinc fingers are joined together to create a DNA-binding domain with specificity of 9–18 base pairs per ZFN monomer [88]. The *FokI* endonuclease domain must dimerize to be functional and will introduce DSB non-specifically at a fixed distance of 9 and 13 nucleotides downstream of the recognition site [89]. Thus, ZFNs are used in pairs with specificity to opposing DNA strands that assemble on both sites of the targeted DNA cleavage site. Resolution of ZFN-induced DSBs via non-homologous end joining (NHEJ) generates small insertions and deletions that often produce null or hypomorphic alleles [86]. The development of ZFNs has provided, for the first time, an efficient and relatively simple platform for inducing site-specific mutations, particularly for non-murine model organisms for which this technology was lacking. Heritable, ZFN-induced mutagenesis has been shown to be effective in several vertebrate species including zebrafish, rats, rabbits, and pigs, where the frequency of target modification can reach 10% [90]. Recently, *X. tropicalis* has entered the growing list of animal models for which mutants can be generated by targeted mutagenesis with ZFNs [91–93] (Table 3). ZFNs directed against the *noggin* (*nog*) gene produced founder animals carrying deletions and insertions at a frequency of up to 47% that were transmissible to the next generation [91,93]. ZFNs were also used to generate albino lines of *X. tropicalis* by targeting the *tyrosinase* (*tyr*) locus [92]. Surprisingly, vitiligo was observed in the skin of F0 founder frogs indicating mosaicism caused by disruption of both copies of the *tyr* genes in some melanophores. Finally, targeting the *pft1a* locus was achieved with a mutation rate of 9.8% [91].

ZFN are clearly powerful tools for gene editing and their DNA-binding specificities are relatively predictable, but several problems are associated with this technology [94] (Table 2). For example, context-dependent effects among individual fingers in an array have to be taken into account when engineering of ZFN [95]. Combining multiple zinc fingers to target unique sites in the genome is therefore an

Table 3
Examples of genes targeted by ZFN and TALEN-mediated genome editing in *Xenopus*.

Nuclease	Species	Gene	Somatic mutation rates[a]	Germline transmission	References
ZFN	*X. tropicalis*	eGFP transgene	24%	N.D.	[93]
ZFN	*X. tropicalis*	*nog*	10–47%	Yes	[93]
ZFN	*X. tropicalis*	*nog*	17.4%	N.D.	[91]
ZFN	*X. tropicalis*	*ptf1a*	9.8%	N.D.	[91]
ZFN	*X. tropicalis*	*tyr*	N.D.	Yes	[92]
TALEN	*X. tropicalis*	*atp6v0a1* (*vpp1*)	87.0%	N.D.	[91]
TALEN	*X. laevis*	eGFP transgene	85.7%	N.D.	[112]
TALEN	*X. tropicalis*	*ets1*	88.9%	Yes	[91]
TALEN	*X. laevis*	*ets1.a*	86.9%	N.D.	[91]
TALEN	*X. tropicalis*	*foxd3*	95.7%	N.D.	[91]
TALEN	*X. tropicalis*	*hhex*	82.6%	N.D.	[91]
TALEN	*X. tropicalis*	*hspa5* (*grp78*/*bip*)	61.9%	N.D.	[91]
TALEN	*X. tropicalis*	*mmp9* (*mmp-9th*)	35.7%	N.D.	[110]
TALEN	*X. tropicalis*	*ndrg1a*	90.3%	N.D.	[114]
TALEN	*X. tropicalis*	*nog*	90.0%	N.D.	[91]
TALEN	*X. tropicalis*	*nog*	81.2%	N.D.	[110]
TALEN	*X. laevis*	*npm3.a*	100%	N.D.	[115]
TALEN	*X. laevis*	*npm3.b* (*no29*)	90%	N.D.	[115]
TALEN	*X. laevis*	*pax6.a*	100%	N.D.	[113]
TALEN	*X. laevis*	*pax6.b*	100%	N.D.	[113]
TALEN	*X. tropicalis*	*ptf1a*	80.3%	Yes	[91]
TALEN	*X. tropicalis*	*sox9*	85.0%	N.D.	[91]
TALEN	*X. tropicalis*	*tyr*	70%	Yes	[109]
TALEN	*X. tropicalis*	*tyr*	12.5%	N.D.	[110]
TALEN	*X. tropicalis*	*tyr*	100%	N.D.	[111]
TALEN	*X. laevis*	*tyr.a*	50%	N.D.	[113]
TALEN	*X. laevis*	*tyr.a*	100%	N.D.	[115]
TALEN	*X. laevis*	*tyr.b*	100%	N.D.	[115]

Gene symbols are listed according to the *Xenopus* Gene Nomenclature Guidelines (www.xenbase.org). In cases where the referenced studies use alternative symbols, they are indicated in parentheses. N.D., not determined.

[a] Mutation rates were determined by PCR assays on genomic DNA of pooled founder embryos and examination by DNA sequencing. The rates are defined as the ratio of mutant amplicons to total amplicons sequenced.

empirical process that requires laborious screening and optimization. Due to these constraints, not all genes in a model organism genome can be adequately targeted with ZFNs. Furthermore, besides targeting the intended sites, unexpected cleavage effects have also been observed in vivo [96,97]. Unfortunately, these cannot be predicted using conventional in silico analysis. Off-target cleavage by ZNFs can cause cytotoxicity and has raised questions about the specificity of ZFNs. Finally, the intellectual property for ZFNs has been closely guarded by a single biotech company, Sangamo, and its commercial partner Sigma-Aldrich, making access to the technology costly. Given these limitations and restrictions, the initial enthusiasm for ZFNs has faded to a growing need for alternative genome modification technologies.

4.4. Transcription activator-like effector nucleases (TALENs)

TALENs have recently emerged as an alternative research tools to ZFNs for genome editing. The design of TALENs is simple, they harbor robust activity and the gene targeting range is nearly unlimited. Similar to ZFNs, TALENs are artificial DNA nucleases that are engineered to consist of a custom-designed DNA binding domain, known as the transcription activator-like (TAL) effector domain, fused to the catalytic domain of the endonuclease *FokI* [98,99]. TAL effectors are proteins synthesized by phytopathogenic bacteria of the genus *Xanthomonas*, which are injected into plant cells, where they bind to the host genome to activate gene expression and cause disease symptoms [100]. The TAL effector recognition domain is composed of an N-terminal domain that recognizes a 5′ T in the binding site, followed by a variable number of highly conserved 33–35 amino acid repeats each capable of specifying a single DNA nucleotide, and terminated by a C-terminal sequence taken from naturally occurring TAL effector proteins. The different DNA-binding specificities of each repeat are determined by two amino acids known as repeat variable di-residues or RVDs. For each of the four DNA nucleotides, repeats bearing different RVDs have been identified and hence there is a simple one-to-one correspondence of RVD per nucleotide [101,102]. These DNA-binding domain repeats can be assembled into arrays capable of binding essentially any nucleotide sequence in the genome that begins with a 5′ T [103,104]. Whereas TALENs with a wide range of RVDs (9 to 30-RVDs) have been reported to show DNA-binding activity, a simplified 15-RVD TALEN design seems to be sufficient to ensure high in vivo genome targeting efficiencies [105]. Overall, the remarkable simple and programmable DNA-binding code suggests that TALENs with new specificities are easier to design than ZFNs.

Activation of the *FokI* endonuclease activity requires dimerization. A pair of TALENs is therefore designed to bind to adjacent DNA target sequences. Upon DNA binding, the *FokI* domains dimerize and become activated to generate DSB in the spacer region between the two TALEN binding sites (Fig. 5A). As with ZFNs, TALEN-induced DSB activate DNA repair mechanisms, which can be exploited to generate DNA sequence modifications at or near the cleavage site (Fig. 6). Repair can either occur through error-prone non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathway, leading to either short insertion or deletion (indel) mutations or DNA replacement [59,86]. In eukaryotic cells, NHEJ rejoins the broken ends of the chromosome and represents the primary mechanism by which DSBs are repaired [106] (Fig. 6A). NHEJ is imprecise and error prone as it does not use a homologous template for the repair process. As a consequence, small insertions or deletions (indels) occur at the site of the break, often introducing frame-shifts that disrupt the gene function [106].

The alternative mechanism by with DSBs can be repaired is through HDR, which is a DNA template-dependent repair pathway [106]. HDR faithfully inserts a donor molecule at the targeted locus, if a homology-containing donor template is supplied along with the TALENs (Fig. 6B). This strategy can be exploited to insert a single or multiple transgenes as well as to generate single nucleotide substitutions. In the first case, a TALEN pair is co-delivered with a linear donor sequence bearing locus-specific homology arms to achieve in vivo gene additions. In the second case, co-delivery of TALENs with single-stranded DNA oligonucleotides can be exploited for gene editing by HDR. The

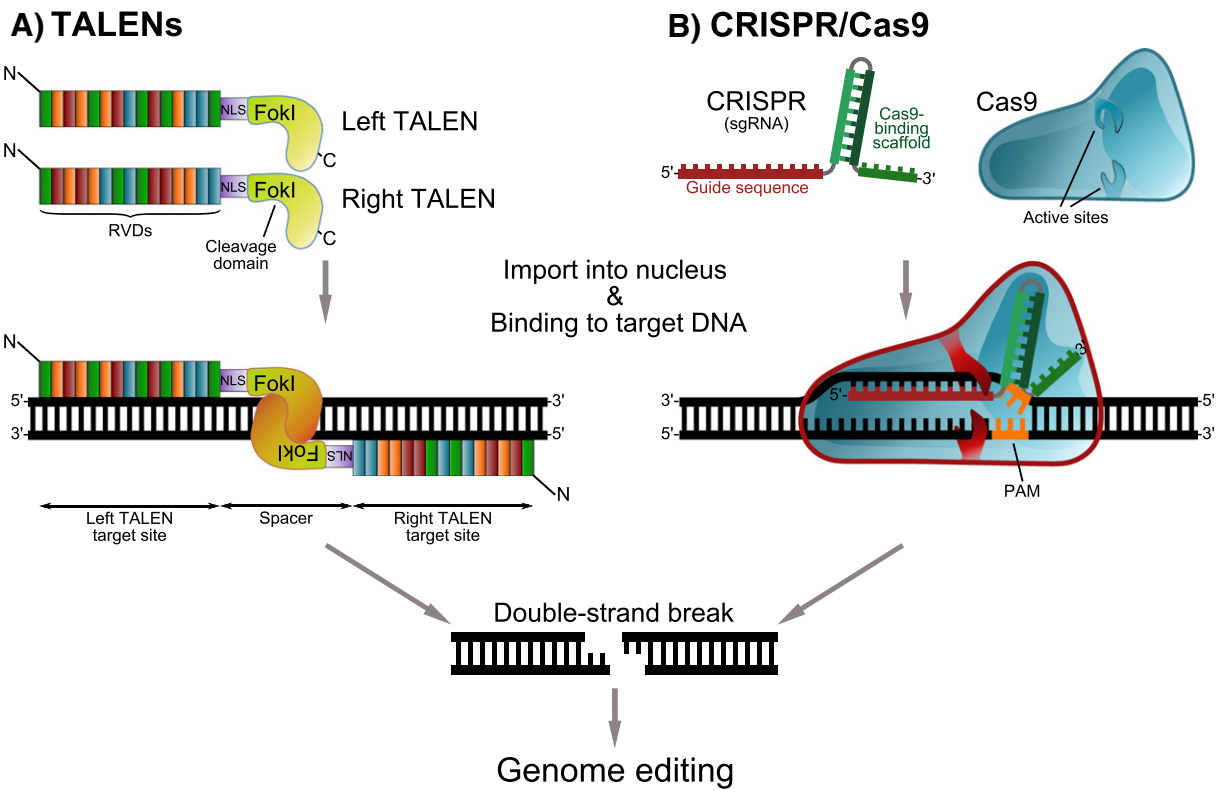

Fig. 5. Genome editing using TALENs and CRISPR/Cas-based methods. A) TALEN-mediated genome editing. TALENs are shown as TAL effector fusions to the catalytic domain of the type IIS restriction endonuclease *FokI*. The native nuclear localization signal (NLS) ensures targeting of the TALENs to the nucleus. TALENs recognize their genome target sequences via a central protein domain composed of variable number of amino acid repeats (each colored separately). Individual TALE repeats contain 33–35 amino acids that recognize a single base pair via two hypervariable amino acid residues (repeat-variable diresidues, RVDs). TALEN target sites (16–17 bp) consist of two TALEN binding sites separated by a spacer sequence of varying length (12–31 bp). Double-stranded DNA breaks are introduced at the target site by dimerization of *FokI*. B) CRISPR/Cas-mediated genome editing. The system consists of two components, a dual nuclear localization signal-tagged Cas9 protein and a single guide RNA (sgRNA). The sgRNA is a chimera composed of a 20-nucleotide guide sequence (*red*) complementary to the target site at its 5′ end and a Cas9-binding scaffold (*green*) at its 3′ end. The guide sequence pairs with the DNA target directly upstream of a requisite protospacer adjacent motif (PAM; 5′-NGG motif; *orange*). The sgRNA will assemble into a complex with Cas9 to introduce site-specific DNA double-strand breaks. The Cas9 endonuclease active sites cut between 3 and 7 nucleotides upstream of the PAM on both strands. Independent of the methodology, the induced double-stranded DNA breaks are repaired by different mechanisms resulting in genome editing (see Fig. 6 for details).

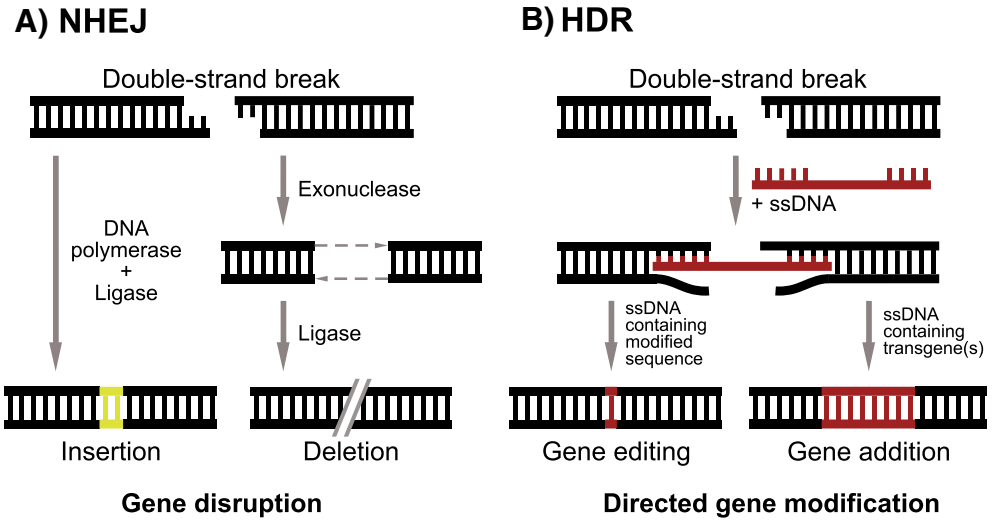

Fig. 6. Nuclease-induced genome editing. Double-stranded DNA breaks introduced by either TALENs or Cas9 can be repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR). A) In the error-prone NHEJ-mediated repair pathway, the DNA breaks are processed by the endogenous DNA repair machinery and rejoined, which can result in random short insertion or deletion (indel) mutations at the target site. Indel mutations occurring within the coding region of a gene can result in frameshifts and the creation of premature stop codons, resulting in gene disruption. B) In the presence of a complementary single-stranded DNA (ssDNA) donor oligonucleotide or a plasmid sequence, HDR leads to two different outcomes. *Left*: If the DNA donor is designed to contain a modification of the target gene, HDR will use the donor template to edit the target gene. *Right*: If the DNA donor contains a transgene and extended homology arms, HDR can be used to introduce single or multiple transgenes to modify or replace the existing gene.

oligonucleotide template will direct the introduction of point mutations, deletions or insertions at the target site. Together, these gene modification approaches can be employed for the study of human gene functions and the modeling of inherited human diseases in non-mammalian model organisms in a previously unprecedented manner.

To date, TALEN technology has enabled the introduction of targeted genome modifications in various vertebrate model organisms, including zebrafish, mice, rats, and livestock [59,100]. For example, TALENs have been used in zebrafish for targeted integration enabling the generation of loxP engineered loci and the possibility for conditional gene activation [107,108]. This opens the exciting possibility to alter gene functions also in older embryos, larvae or adult organisms, particularly in those cases, where the gene of interest has an essential early embryonic function. Furthermore, organ-specific gene functions can be studied using tissue-specific Cre-mediated gene inactivation or inducible gene expression systems. Technically, the delivery of TALEN pairs is simple. Fertilized zebrafish eggs are microinjected with TALEN mRNAs and either single-stranded DNA oligonucleotides or donor plasmids with extended homology arms (>800 bp). The knockin efficiency is however low requiring careful screening of the founder generation. With optimized TALEN designs (15 RVDs, spacer 13–20 bp, 5′ T-nucleotide requirement), the somatic mutations rates in zebrafish embryos can reach >80%, averaging 58 ± 6% [105].

As illustrated in Table 3, *Xenopus* has rapidly joined the collection of vertebrate animal models, whose genomes have been successfully manipulated using TALENs [91,109–114]. The targeting of eight genes in the genome *X. tropicalis* using TALENs resulted in mutation rates ranging 61.9% to 95.7% [91]. The high mutation rates suggested that both alleles were disrupted, which was confirmed by phenotype analysis. Furthermore, the TALEN-induced gene disruptions were heritable as demonstrated for mutations in the *etv1* (formerly *ets1*) and *ptf1a* (formerly *ptf1a*/*p48*) genes. Highly efficient bi-allelic mutations rates in *X. tropicalis* animals were also reported for TALENs targeting the *tyrosinase* (*tyr*) gene, where mutations caused full albinism in more than 70% of the F0 animals [109–111]. Given the high mutation rates, TALENs have also been used as an independent gene disruption tool to confirm phenotypes obtained by antisense MOs, as illustrated by a recent study of the *ndrg1a* gene in *X. tropicalis* [114].

Despite its paleotetraploid genome, targeted gene disruptions by TALENs were also found to be highly efficient in *X. laevis* embryos [91,113,115]. For example, mosaic but biallelic *tyr* gene disruption by frameshift mutations resulted in retinal pigment epithelia and melanophores devoid of pigment [113,115]. Mutation rates were up to 86.9% (*etv1.a*), 90% (*npm3.b*/*no29*) and 100% (*npm3.a*, *pax6.a*, *pax6.b*, *tyr.a*, *tyr.b*) indicating that testing of multiple TALEN pairs targeting different sites for a given locus will be necessary to achieve maximal mutation rates (Table 3). In the case of the *tyr* genes, the TALEN target sites were conserved between the two *tyr* homeologs and thus a single TALEN pair could be used resulting in the same mutagenesis efficiencies for *tyr.a* and *tyr.b* [115]. For the *npm3* genes, this was not possible. Here, two different TALEN pairs were coinjected and mutations in both homeologs (*npm3.a*, *npm3.b*/*no29*) were introduced with efficiencies between 90–100% [115]. Overall, these studies indicate that TALENs can target most genes in the genomes of *X. tropicalis* and *X. laevis*. Furthermore, potential redundancies resulting from the presence of two homeologs in the *X. laevis* genome can be overcome by targeting both homeologs simultaneously.

How do TALENs compare to ZFNs as gene editing tools in *Xenopus* embryos? In a side-by-side comparison of TALENs and ZFNs in *X. tropicalis* focusing on the *nog* and *ptf1a* genes, it was possible to reach mutagenesis rates between 80–90% with TALENs and the embryos could tolerate high doses of TALEN mRNAs [91] (Table 3). By contrast, targeted mutagenesis rates with ZFNs did not exceed 15–17% as injection of higher doses of ZFN mRNAs led to high proportions of abnormal and dead embryos. An examination of potential TALEN off target sides in the *X. tropicalis* genome failed to detect any mutations by DNA sequencing [91]. In zebrafish, recent comparisons of the mutation efficiencies of ZNFs and TALENs also supported the superiority of TALENs over ZFNs [105,116]. For example, a large-scale comparison of ZFN and TALEN mutagenicity involving 84 ZFN pairs targeting 66 genes and 34 TALEN pairs directed against 18 genes revealed that TALENs induce an average of 10-fold more specific mutations than ZFNs [116]. For both animal models, lower toxicities and comparable or higher somatic mutation activities were reported using TALENs harboring an obligate heterodimeric *FokI* nuclease domain instead of homodimeric *FokI* [111,117,118]. Taken together, the higher mutation rates, lower off-target effects, and the extended versatility in targeting any sequence in the genome make TALENs more superior over ZFNs for targeted genome mutagenesis in non-mammalian model organisms.

4.5. CRISPR/Cas system

Despite the growing popularity of site-directed artificial nucleases, particularly TALENs, for genome editing, a newly developed technology on the basis of the bacterial CRISPR/Cas system has recently emerged and may have significant advantages [59,119,120]. In bacteria and archaea, the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated (Cas) system induces adaptive immune response against invading phages and plasmids [121]. Small segments of foreign DNA from an earlier infection are incorporated into the CRISPR loci of the host organism. RNA transcripts from the CRISPR loci are processed into short CRISPR RNAs (crRNAs), which in turn anneal to an auxiliary trans-activating crRNA (tracrRNA) and direct sequence-specific silencing of foreign nucleic acids by Cas endonucleases. In a seminal study, it was demonstrated that a synthetic single guide RNA (sgRNA) of about 100 nucleotides consisting of the fusion of the specificity-determining crRNA and tracrRNA can direct Cas9 endonuclease cleavage of target DNA in vitro [122]. By designing sgRNA to target specific nucleotide sequences, the CRISPR/Cas system can be employed to cleave virtually any DNA sequence in the genome. As shown in Fig. 5B, the 5′ end of a customized sgRNA recognizes a 20-nucleotide target sequence that must be followed by the conserved protospacer adjacent motif (PAM) sequence (NGG for Cas9, where N can be any nucleotide). The remaining 80 nucleotides of the sgRNA form a stem-loop structure that serves as a binding site for Cas9 and recruits the endonuclease to the target sequence. Cas9 contains two separate nuclease domains each responsible for cleavage of one of the two DNA strands in the target site. Thus, unlike ZFNs and TALENs, Cas9 generates DSBs as a monomer without the need for dimerization (Fig. 5). The DSB occurs several base pairs upstream of the PAM sequence (Fig. 5B). As with ZFNs and TALENs, these lesions are imperfectly repaired via the error-prone NHEJ pathway often resulting in the loss or addition of nucleotides, causing frameshift mutations or early stop codons and ultimately loss of function of the targeted gene (Fig. 6). Alternatively, following the cleavage of DNA, a repair fragment, containing for example a reporter gene, flanked by sequences homologous to the cleavage site can be exploited for gene replacement purposes. The donor fragment will act as a template for HDR and result in gene insertion into the target site (Fig. 6). The frequency of HDR is however generally low, as DSB repair is at least tenfold more active in vivo [108]. To decrease the frequency of NHEJ and increase the relative frequency of HDR, Cas9 endonucleases have been engineered into nickases by selectively mutagenizing one of the two nuclease domains. These modified Cas9 endonucleases will introduce a nick into one DNA strand but they are unable to create DSBs as monomers [123,124]. In the presence of a donor construct, the nicks are preferentially repaired by HDR. In addition to enabling a further level of regulation over the DNA repair processes, Cas9 nickases have been used in a double-nicking approach with paired sgRNAs, similar to the ZFN- and TALEN-based genome editing systems, to improve the specificity of CRISPR/Cas-mediated genome editing [124,125]. Together, the recent developments have improved the ability and precision of the

CRISPR/Cas system to generate targeted site-specific mutations in the genome.

The attractiveness of the CRISPR/Cas system for gene modification stems from its simplicity and its ability to do so also in model organisms that have been recalcitrant to genetic manipulation. Target gene modifications are straight forward and require in its simplest conception only the introduction of two molecules into the fertilized egg, blastocyst or tissue culture cell: the Cas9 enzyme, typically as mRNA, and the sgRNA specific to the sequence to be targeted. To date, CRISPR/Cas-mediated genome editing has been successfully applied to various vertebrate model systems, including human cells [123,124,126], mouse [125,127–129], rat [127,130], and zebrafish [131–134]. In 2013, the first studies appeared demonstrating that the CRISPR/Cas system is also effective at modifying the *X. tropicalis* genome [135,136] (Table 4). Targeted mutagenesis of the *tyr* locus by the coinjection of specific sgRNA and mRNA encoding a human codon-optimized Cas9 resulted in biallelic loss of gene function in a large number of cells of the *Xenopus* embryos recovered [135]. Analysis of the *tyr* target site revealed indels typical of NHEJ-mediated DNA repair. Similar conclusions regarding the *tyr* locus were reached in a second independent study, where the albino phenotype was observed in essentially 100% of the injected embryos using two different sgRNAs [136]. Both studies also reported successful germline transmission of the CRISPR/Cas-induced mutations at the *tyr* locus to the F1 generation [135,136]. Targeting a second locus, the *six3* gene, resulted in *X. tropicalis* embryos with eye and anterior head abnormalities similar to the known MO-knockdown phenotypes in *X. laevis* embryos [136]. In a third, more comprehensive study, sgRNAs targeting 10 different *X. tropicalis* genes were designed and tested [137] (Table 4). Precise targeting of all ten genes was achieved. Interestingly, mutation rates above 70% were observed with seven of the ten targeted genes. With additionally designed sgRNAs, the mutation rates could be boosted for all genes to above 75%. Efficient germ-line transmission of the targeted *elastase* and *tyr* mutations was demonstrated by analysis of the F1 generation. Side-directed mutagenesis of two sgRNA revealed that perfect matches between the spacer and protospacer sequences proximal to the PAM were essential for efficient Cas9-mediated cleavages at the target sites in the *X. tropicalis* genome. Multiplexed gene targeting, i.e. simultaneous disruption of multiple genes, by the coinjection of two different sgRNAs was possible with mutation rates comparable to individually injected sgRNAs [137]. Overall, the current evidence suggests that the CRISPR/Cas is an easy, economic, and reliable method for genome editing in *X. tropicalis*. The successful demonstration of CRISPR/Cas-mediated genome editing in *X. laevis* remains to be established, but is being pursued by a number of *Xenopus* laboratories including ours. The demonstration of reliable multiplex genome editing with CRISPR/Cas [137] is particularly important for *X. laevis*, where homeologs may fulfill redundant functions and require the generation of double knockouts.

Potential weaknesses and limitations of the CRISPR/Cas system exist. For example, the requirement for the downstream 5′-NGG-3′ PAM sequence has to be respected. The PAM requirement does not severely limit the targeting range of the CRISPR/Cas system as appropriate target sites can be found on average every 8–12 nucleotides in the human genome [123]. However, additional sequence restrictions apply as sgRNAs need to be synthesized in vitro prior to injection into embryos [132]. Specifically, the sequence constraints of the minimal promoter of the T7 RNA polymerase have to be respected to ensure accurate and efficient in vitro synthesis of sgRNAs. The T7 RNA polymerase introduces mandatory nucleotides at position +1 and +2 of the RNA transcripts, which have to be a guanine or adenine. The potential genomic target sites will therefore have to be compatible with the motif 5′-(G/A)-(G/A)-N_{18}-NGG-3′, which would occurs on average every 32 nucleotides in random DNA. The most optimal motif 5′-GG-N_{18}-NGG-3′ is only found every 128 nucleotides. Due to these technical requirements, the number of potential target sites for sgRNAs in the genome is in reality strongly reduced.

In addition to the targeting range, a number of recent in vitro studies conducted using cell lines have indicated that there are significant off-target mutation rates with the CRISPR/Cas system [138]. By contrast, in vivo studies in model organisms [133,139,140] including *X. tropicalis* [135–137] seem to provide no significant evidence of such undesirable mutations. The higher levels of off-target mutation rates in cell cultures may be caused by the plasmid-driven sustained expression of CRISPR/Cas components, whereas RNA injections are used for in vivo mutagenesis in vertebrate embryos. A careful choice of target sites that lack homologous sequences elsewhere in the genome should be useful in avoiding unwanted off-target mutations. Furthermore, the double-nicking approach using Cas9 nickases and paired sgRNAs can be employed to reduce off-target effects [124,125]. Finally, rare off-target mutations in the founders can be removed by mating.

4.6. Transgenesis

Transgenic embryos expressing in *trans* a gene of interest under the control of a tissue-specific promotor complement the tool box available to study gene functions in *Xenopus* [141]. Pioneered by Kroll and Amaya [142], restriction enzyme mediated integration represents is the most reliable method to generate transgenic *Xenopus* embryos. The method relies on the transplantation of transgenic sperm nuclei into unfertilized eggs and may involve an antibiotic selection step to eliminate non-transgenic embryos [142,143]. The F0 transgenic embryos are non-chimeric and can therefore be analyzed with the need for further

Table 4
Examples of genes targeted by CRISPR/Cas-mediated genome editing in *Xenopus*.

Nuclease	Species	Gene	Somatic mutation rates[a]	Germline transmission	References
CRISPR/Cas	*X. tropicalis*	*elastase*	100%	Yes	[137]
CRISPR/Cas	*X. tropicalis*	*ets1*	76.9%	N.D.	[137]
CRISPR/Cas	*X. tropicalis*	*ets2*	100%	N.D.	[137]
CRISPR/Cas	*X. tropicalis*	*hhex*	100%	N.D.	[137]
CRISPR/Cas	*X. tropicalis*	*hspa5* (*grp78*/*bip*)	88.9%	N.D.	[137]
CRISPR/Cas	*X. tropicalis*	*pdx1*	75%	N.D.	[137]
CRISPR/Cas	*X. tropicalis*	*pgat* (*pat*)	78.9%	N.D.	[137]
CRISPR/Cas	*X. tropicalis*	*ptf1a*	72.2%	N.D.	[137]
CRISPR/Cas	*X. tropicalis*	*six3*	100%	N.D.	[136]
CRISPR/Cas	*X. tropicalis*	*tm4sf4*	100%	N.D.	[137]
CRISPR/Cas	*X. tropicalis*	*tyr*	100%	Yes	[136]
CRISPR/Cas	*X. tropicalis*	*tyr*	84.2%	Yes	[135]
CRISPR/Cas	*X. tropicalis*	*tyr*	82.4%	Yes	[137]

Gene symbols are listed according to the *Xenopus* Gene Nomenclature Guidelines (www.xenbase.org). In cases where the referenced studies use alternative symbols, they are indicated in parentheses. N.D., not determined.

[a] Mutation rates were determined by PCR assays on genomic DNA of pooled founder embryos and examination by DNA sequencing. The rates are defined as the ratio of mutant amplicons to total amplicons sequenced.

breeding, which dramatically cuts the time to complete experimental studies. Transgenic approaches are particular useful to model autosomal dominant human congenital diseases, which often result from gain of function mutations. The mutant gene product is either toxic or exerts a dominant-inhibitory effect over the wild-type protein.

In the context of the present review, transgenic *Xenopus* models of inherited human diseases are of particular interest. Retinitis pigmentosa (RP) is a genetically heterogeneous disorder involving photoreceptor death and progressive loss of visual function [144]. More than 100 genes are linked to RP, which can show autosomal recessive, autosomal dominant, or X-linked patterns of inheritance. Understanding the different pathogenic pathways leading from RP disease gene mutations to photoreceptor death is crucial for the development of RP therapies. Mutations in the *PRPH2* (also known as RDS) gene can cause various human retinopathies, including autosomal dominant retinitis pigmentosa 7 (RP7, OMIM #608133), a late-onset retinal degenerative disease characterized by night blindness, progressive loss of vision, and photoreceptor cell death [145]. PRPH2 encodes for pheripherin-2, a member of the tetraspanin family of membrane proteins that plays a critical role in the morphogenesis of photoreceptor outer segment disks. This is underscored by well over 40 different mutations in the PRPH2 gene. To understand the pathogenic mechanisms underlying different PRPH2 mutants, Loewen et al. [146] generated transgenic *Xenopus* tadpoles expressing different autosomal dominant RP7-linked PRPH2 mutants. *X. laevis prph2* mutants analogous to the human PRPH2 mutants were expressed in rod photoreceptors as transgenes under the control of the *X. laevis* rhodopsin promoter. Interestingly, the prph2^{P216L} mutant caused photoreceptor degeneration through a dominant-negative mechanism [146]. Hence, the transgenic prph2^{P121L} *Xenopus* mutant represents the first *Xenopus* model of human autosomal dominant RP17. The most common form of autosomal-dominant RP is attributed to missense mutations in the rhodopsin (RHO) gene and accounts for about a third of the reported cases [147,148]. Rhodopsin-related RP is classified as RP4 (OMIM #613731), which characterized by early onset of night blindness and abnormal fundus. Rhodopsin, a G-protein coupled receptor, is the visual pigment of rod photoreceptors and mediates vision in dim light. It is composed of an opsin apoprotein with the covalently coupled vitamin A-derived chromophore 11-*cis* retinal. RHO^{P23H} represents the most common autosomal dominant RHO mutation found in RP4 patients in North America and involves a single amino acid change in the highly conserved proline residue at position 23 [149]. Different laboratories have developed transgenic *X. laevis* tadpoles expressing the P23H mutant [150–152]. As in mammalian RHO^{P23H} models, severe rod photoreceptor degeneration resembling RP was observed. Other RHO mutants modeled in transgenic *Xenopus* embryos include mutations that affect the C-terminus of rhodopsin causing the most severe forms of RP [153,154] and those disrupting glycosylation of rhodopsin, which is responsible for a subset of RP known as sector RP in which the inferior retina preferentially degenerates [155]. Despite of their success as illustrated with the examples given above, classical transgenic approaches are likely to be replaced in the future by TALEN or CRISPR/Cas mediated genome editing strategies that enable the precise introduction of specific autosomal dominant mutations within the correct native genomic context.

4.7. General considerations

Tables 3 and 4 summarize the present state of research regarding the application of the novel ZFN, TALEN, and CRISPR/Cas-based genome engineering methods in *Xenopus*. It is evident that TALEN and CRISPR/Cas are superior to the ZFN methodology for gene editing in *Xenopus* as somatic mutation rates are high, typically between 80–100%. As elaborated above, antisense MOs, TALENs, and CRISPR/Cas represent currently the three main strategies to specifically modify gene functions in *Xenopus* embryos. Each methodology has its advantages and disadvantages (Table 2). When planning morpholino-mediated gene knockdowns or gene editing in *X. laevis*, it is important to take into account that its genome is paleotetraploid. Hence, two closely related genes, known as homeologs, need to be taken into consideration. In general, the sequences of a pair of homeologous genes share a high degree of similarity and they typically encode functionally equivalent proteins. In many cases, it is possible to design a single MO or sgRNA, or a pair of TALENs to target both homeologs. Due to small sequence differences, it may however be necessary to use two MOs, sgRNAs or TALEN pairs. The decision which strategy to be adopted is also dependent on the biological question being studied. For example, functional redundancies of *irx* paralogs during kidney organogenesis were rapidly assessed using simultaneous MO knockdowns [156]. This result would be difficult and time consuming to establish by crossing genetic mutants. TALENs are highly efficient at generating bi-allelic somatic mutations in F0 embryos of both *Xenopus* species [91,109,113], it appears however that the penetrance of mutant phenotypes is not as profound as with MO knockdowns. On average 2/3 of the TALEN induced indel mutations will result in reading frame shifts, which have to occur in a bi-allelic manner to insure the generation of homozygous null alleles in the F0 generation. Moreover, TALENs cannot block functions of maternal mRNAs, which is however possible with MOs. Similar restrictions apply to CRISPR/Cas-mediate genome editing methodologies. This suggests that, at present, MOs cannot be completely replaced by TALENs or CRISPR/Cas to generate F0 embryos with fully penetrant mutant phenotypes. These considerations need to be taken into account, when one is planning to employ mutant *Xenopus* embryos for drug discovery screening.

The attractiveness of the CRISPR/Cas system lies in the fact that the investigator only needs to synthesize a new 100-nucleotide sgRNA to target the Cas nuclease to a new genome target site. This allows the rapid generation of hundreds to thousands of new target-directed nucleases. By contrast, the ZFN and TALEN technologies require the engineering of two DNA-binding nucleases for each new target site. Furthermore, the assembly process is significantly more complex than the one for the CRISPR/Cas system as it requires libraries consisting of several dozen plasmids. The CRISPR/Cas system greatly simplifies the design and lowers the costs of gene targeting compared to the protein-based gene targeting technologies. Recent methodological advances indicate that highly efficient gene knockouts can be achieved in embryos by injection of sgRNA bound to recombinant Cas9 protein resulting in germ-line transmission in up to 93% of mutant embryos with minimal toxicity [140]. In a separate study, a novel, highly efficient method for CRISPR/Cas-mediated gene knock-ins was reported that is based on homology-independent DSB repair [139]. After the coinjection of a circular donor plasmid with a sgRNA and Cas9 mRNA, concurrent cleavage of the donor plasmid and the chromosomal integration site resulted in target integration of the donor DNA with at least ten-fold higher rates than when performing knock-ins by HDR. Both methods expand the repertoire of genome editing possibilities in non-murine model organisms. For example, it will be possible to use CRISPR/Cas-mediated knock-in methods to engineer *Xenopus* mutants carrying a gain-of-function mutation mimicking autosomal dominant traits in human patients suffering from rare inherited diseases.

5. Small molecule screening in *Xenopus*

5.1. The impact of zebrafish

The first large-scale application of chemical screening employing vertebrate embryos was reported by Peterson and colleagues in 2000 [157]. Using zebrafish embryos, they examined the effects of 1100 synthetic small organic molecules on embryonic development by visual inspection under the dissection microscope. The vast majority of the compounds were inactive, but 1% of the molecules tested were found to selectively perturb the development of the central nervous system, ear, heart, and skin pigmentation. These bioactive compounds could

be very potent with effects observed already at nanomolar concentrations and they acted specifically as frequently only one organ was affected. The precise cellular targets of the recovered active compounds remain unknown to date. Nevertheless, this seminal study demonstrated that free-living vertebrate embryos are permeable to many small organic molecules underscoring their value as high-throughput in vivo drug screening system.

Building on the pioneering work done with zebrafish embryos by Peterson and colleagues [157], it was later shown that many of the active compounds recovered in the zebrafish screen induced similar phenotypes when applied to *Xenopus* embryos [17]. Subsequently, three large-scale phenotypic drug screens using *Xenopus* embryos were successfully carried out [158–161]. The key features of these screens are shown in Table 4 and will be discussed below in detail. Collectively, these studies firmly establish *Xenopus* as a second powerful non-mammalian animal model for phenotypic drug discovery screens.

5.2. Whole-organism based phenotypic screens in Xenopus

The simplest phenotypic screening strategies are those that do not require labor-intensive and time-consuming down-stream processing of the treated embryos prior to scoring potential hits. By inspecting embryos with a low-magnification dissecting microscope, the effects of compound treatment on organ development are easily and noninvasively monitored. As development proceeds *Xenopus* embryos gain progressively transparency by metabolizing yolk platelet. This in turn facilitates the observation of a range of organs and tissues, including the heart, eyes, inner ears, skeletal musculature, pigment cells, and the vasculature.

Changes in pigmentation patterns are particularly easy to score in *Xenopus* embryos (Fig. 1C). Pigment cells (melanophores in amphibians; melanocytes in mammals) arise from neural crest progenitor cells and they can transform to cause melanoma, highly malignant tumors of melanocytes. Chemicals that suppress pigment cell development in vivo may therefore become useful for treating melanoma. On this premise, Tomlinson and colleagues screened 2990 organic molecules to identify those that selectively disrupt pigment cell development in *Xenopus* embryos [161]. A total of 29 compounds were recovered that either caused loss of pigmentation, affected pigment cell morphology, or interfered with pigment cell migration. Further analysis of the 8-quinolol derivate NSC84093, a selective inhibitor of pigment cell migration, revealed that it exerts its inhibitory effects by targeting matrix proteinases (MMPs) [160]. Importantly, this discovery implicated a role for MMPs in regulating pigment cell migration in vivo, which was subsequently confirmed by MO-mediated knockdown studies in *Xenopus* embryos [160]. Another compound, NSC210627, suppressed pigment cell formation in *Xenopus* and zebrafish [162]. NSC210627 shares structural similarity with brequinar, an inhibitor of dihydroorotate dehydrogenase (DHOH) [163], implicating DHOH as a novel target for melanoma drug development. Indeed, the structurally unrelated DHOH inhibitor leflunomide phenocopies the pigment cell defects induced by treatment of embryos with NSC210627 [162]. Subsequent studies with leflunomide, a well-tolerated drug approved for the treatment of arthritis, demonstrated that it acts as an inhibitor of both human melanoma cell proliferation in vitro and in mouse xenograft models [162]. This suggested that leflunomide might be effective for the treatment of patients suffering from metastatic melanoma. In fact, a Phase I/II clinical trial was initiated in 2012 to test whether a combination therapy of leflunomide with the BRAF inhibitor vemurafenib would benefit melanoma patients (Clinical trial ID number: NCT01611675) [11]. Leflunomide is therefore the first drug candidate originating from phenotypic drug screening to enter clinical testing. Unfortunately, participant recruitment for the clinical trial was suspended in December 2013 for undisclosed reasons. Despite this recent set-back, the example of NSC210627 is instructive as it provides the blue print for phenotypic drug discovery screening in non-murine embryos resulting in the repurposing of an approved drug leflunomide for a novel indication in cancer therapy.

An alternative approach to identify drug candidates for cancer therapy by screening in *Xenopus* embryos was described by Kälin and colleagues [159]. Angiogenesis and lymphangiogenesis do not only play an important role during embryonic development and organogenesis, but are also implicated in the pathogenesis of tumor progression and chronic inflammation [164,165]. Disruption of blood or lymph vessel development in *Xenopus* embryos results in fluid accumulation and edema formation. Using edema formation as an easily scorable pathophysiological readout, a two-tiered phenotypic screening protocol was developed to identify compounds with anti-angiogenic and/or anti-lymphangiogenic activities in *Xenopus* embryos. In a first step, a commercial library of 1280 well-annotated chemical compounds was screened to identify compounds capable of inducing edema in *Xenopus* embryos. In the second step of the protocol, whole mount in situ hybridization was used to identify the subset of edema-inducing drug candidates that interfere with blood and/or lymphatic vessel development. Using this protocol, 32 compounds interfering with various aspects of vascular development were recovered [159]. Positive hits included known inhibitors of mammalian angiogenesis, such as vascular endothelial growth factor (VEGF) receptor antagonists, which validated the in vivo screening approach. Several novel antiangiogenic compounds are identified, which are now subject to detailed characterization to determine their molecular targets and to evaluate their potential as antiangiogenic drugs in mammalian animal models. Interestingly, the pathophysiological screening protocol with *Xenopus* embryos was found to be more sensitive at recovering antiangiogenic compounds than one employing transgenic zebrafish expressing a fluorescent vascular reporter gene [166]. In a proof-of-principle study, one of the compounds identified in the *Xenopus* screen, an adenosine receptor antagonist, was shown to block VEGF-induced neovascularization in adult mice [159]. This indicates that drug candidates recovered from phenotypic drug discovery screens in *Xenopus* embryos retain comparable bioactivities when tested in mammalian models. Studies involving the in vivo testing for potential anti-cancer activities using tumor cell xenografts transplanted into nude mice are in progress and will ultimately decide whether the drug candidate has the potential to enter clinical testing in the future.

The phenotypic *Xenopus* drug screens described so far relied on commercial chemical libraries, which were enriched for compounds with drug-like features and well-defined pharmacological properties. Dush and colleagues (2011) have described a screening approach that combines the advantages of whole organism testing and multiplex profiling of chemical compounds to discover small molecules that interfere with the establishment of left-right asymmetries of organ positioning in *Xenopus* embryos [158]. The correct anatomical placing of the lungs, heart, pancreas, liver, and other vital organs during embryogenesis is essential for the proper functioning of the cardiovascular system, the respiratory organs, and the digestive tract in the adult [167,168]. The process is dependent on the exclusive expression of TGF-β signaling components on the left side of the vertebrate embryo. Disruption of left-right asymmetry results either in partial deviations (*heterotaxia*), which causes life-threatening birth defects, or complete reversal of organ placement (*situs inversus*). In *Xenopus* embryos, unlike zebrafish, the left-right asymmetries of organ positions is established in a process highly analogous to humans and can be easily scored by examining the looping of heart and intestines. On this basis, *Xenopus* embryos were employed in a phenotypic screen for heterotaxia-inducing compounds to identify a novel class of pyridines with TGF-β inhibitory activity [158]. A solid-supported multicomponent cyclomerization reaction was used to generate a pilot library of 130 novel compounds as 44 pools of regioisomers, which were examined for heterotaxia-inducing activity in *Xenopus* embryos. One pyridine pool induced clear heterotaxia in 100% of the embryos, which led to the purification of the active regioisomer named heterotaxin. Further analysis of this

molecule revealed that it elicits multiple TGF-β-dependent phenotypes throughout development, including pertubation of melanogenesis and inhibits TGF-β dependent intracellular signaling events [158]. The effects on pigment cells include a striking increase in pigmentation and dendricity, and a dose-dependent reduction in the number of abdominal melanophores. Although the direct protein target of heterotaxin was not determined, it appears to act upstream of Smad2 phosphorylation defining heterotaxin as a novel TGF-β signaling inhibitor. Heterotaxin may therefore have therapeutic potential as an inhibitor of pathologic conditions mediated by excess TGF-β signaling, such as in fibrotic processes and metastatic cancers.

5.3. Logistics of chemical screening with vertebrate embryos

While the present body of work has firmly established that chemical screens can be performed in *Xenopus* embryos with comparable or better results as in zebrafish, there are a number of practical advantages in the handling of *Xenopus* over zebrafish embryos. High-throughput screening of chemical libraries requires large numbers of developmentally synchronized embryos [6,13,169,170]. They are typically arrayed in groups of at least 5 embryos in 48- or 96-well plates prior to the application of chemical compounds. A zebrafish mating pair will generate up to 300 fertilized embryos (Table 1), which would be sufficient to test about 50 compounds at a single concentration. This limitation can be overcome by using mass breeding vessels, but these require dozens of mating pairs to generate thousands of embryos [171]. The logistics become even more complex with zebrafish strains carrying recessive mutations since only a quarter of the offspring will develop a homozygous mutant phenotype. By contrast, a single *Xenopus* female will typically lay thousands of eggs that can be fertilized in vitro to yield highly synchronized embryos. Another problem arises from the chorion that protects zebrafish embryos during the first 48–72 h of embryonic development. Treatment of embryos with intact chorions raises concerns about compound penetration and it interferes with downstream processing such as whole-mount immunostaining or in situ hybridization procedures [169]. Large-scale dechorination of zebrafish embryo relies on pronase treatment. Unfortunately, embryos lacking their chorions are fragile and contact with plastic or air has to be avoided, which limits their utility for robotic handling. *Xenopus* embryos are larger than zebrafish and generally very robust to handle even at earlier stages. They can be arrayed in 48–96 well dishes, where test compounds are added to the media, typically a simple salt solution. Initially, the highly porous vitelline membrane protects the embryos. Hence, accessibility of waterborne organic molecules to the embryo is usually good. Overall, the logistics and the handling of *Xenopus* embryos for large-scale chemical screening is straight forward requiring less on hand processing and the robustness of *Xenopus* embryos reduces an important unwanted source of variability in a drug screening protocol.

6. Developing *Xenopus* models of rare human inherited diseases

6.1. A need for alternative models of inherited human diseases

Patients suffering from rare inherited diseases have typically no or very limited treatment options available that offer long-term cure of the disease [3]. Impressive advances have been made in the last two decades in deciphering the genetic and pathophysiological mechanisms underlying many inherited human diseases. Complementing these activities, transgenic mouse models of inherited human diseases have been generated and they have become instrumental in both the characterization of normal and abnormal disease genes and in providing novel insights into disease initiation and progression [172]. Despite their strengths, the utility of transgenic mouse models for the development of novel therapeutics to treat rare inherited diseases is however limited to preclinical testing of selected drug candidates as they cannot be employed for large-scale drug discovery screening. In vitro approaches using patient-derived cell lines for the screening of the large million-compound libraries used by the pharmaceutical industry are feasible. They have however their limitations as cell cultures cannot recapitulate all aspects of human inherited diseases, such as pathophysiological mechanisms, drug metabolism, and the interactions between different cell types, tissues or organs. Hence, there is a clear need for reliable non-murine animal models of human inherited diseases that can cover the territory from drug discovery to preclinical testing in mice. Zebrafish and *Xenopus* embryos are small enough and have the logistical advantages required for large-scale drug discovery screens. Furthermore, a host of novel genome editing techniques now enables the precise and rapid engineering of non-murine models of human inherited diseases.

For most cases of rare inherited diseases, the altered protein products of the disease-causing genes are either not drugable or there is no obvious therapeutic strategy evident to bypass the gene deficiencies. The recent example of Kalydeco (ivacaftor, VX-770), a drug recently approved for the treatment of cystic fibrosis in patients carrying the rare autosomal recessive G551D mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, remains one of the few notable exceptions to the rule [173]. CFTR proteins with the G551D mutation are correctly trafficked to the cell surface but are dysfunctional. Kalydeco binds specifically to the G551D CFTR mutant and potentiates chloride transport [174]. The G551D mutation accounts however for only 4–5% of the cases of cystic fibrosis and thus the vast majority of cystic fibrosis cases will not benefit from Kalydeco. For cases of autosomal recessive mutations that result in complete loss of the gene function is typically unclear which therapeutic intervention could best compensate for the lost gene function. Enzyme replacement therapy is an option in some special cases, such as the Gaucher's disease, a lysosomal storage malfunction [3]. Gene therapy is still largely experimental and only an option for severe immunodeficiency disorders [175].

6.2. Phenotypic drug screening with models of rare inherited diseases

In vivo phenotype-based drug discovery screens of compound libraries do not require prior knowledge of the molecular mechanism and/or the protein target that will provide therapeutic benefit [7]. Therefore, this approach can have an important role in drug discovery for many rare inherited diseases, which lack effective drug therapies. The development of ZFN-, TALEN- and CRISPR/Cas-based technologies for genome editing in non-murine model organism enables for the first time the generation of tailor-made models that accurately phenocopy human inherited diseases. Importantly, both traditional knockdown methodology using MOs as well as the novel gene editing methods, which rely on mRNAs encoding the necessary technology components, permit the generation of large numbers of mutant embryos displaying the disease pathologies of human inherited diseases by simple injection into the fertilized zebrafish and *Xenopus* eggs. Irrespective of the employed strategy, the mutant embryos can then be used to identify small organic molecules that can ameliorate the disease manifestations in vivo and thus provide therapeutic benefit to the affected embryos (Fig. 7). MOs are rapid and effective gene knockdown tools. The inhibitory effects of MOs are however transient, lasting only a few days into embryogenesis. They are therefore primarily suitable for generating disease models, where the disease phenotype becomes apparent during embryonic stages. For example, knockdown of *bicc1*, in *Xenopus* embryos causes defects in pronephric kidney function [176,177] and represents a *Xenopus* model of human cystic renal dysplasia (OMIM #601331), a rare disorder conferred by hypomorphic mutations in the BICC1 gene [178]. For later-onset phenotypes, the novel gene editing technologies need to be employed to create targeted mutations in the genome of embryos. These approaches generally require the raising of putative founders to adulthood and outcrossing to identify carriers. In contrast to MO-based approaches, this is time-consuming, labor-intensive, and requires sufficient animal housing capacities.

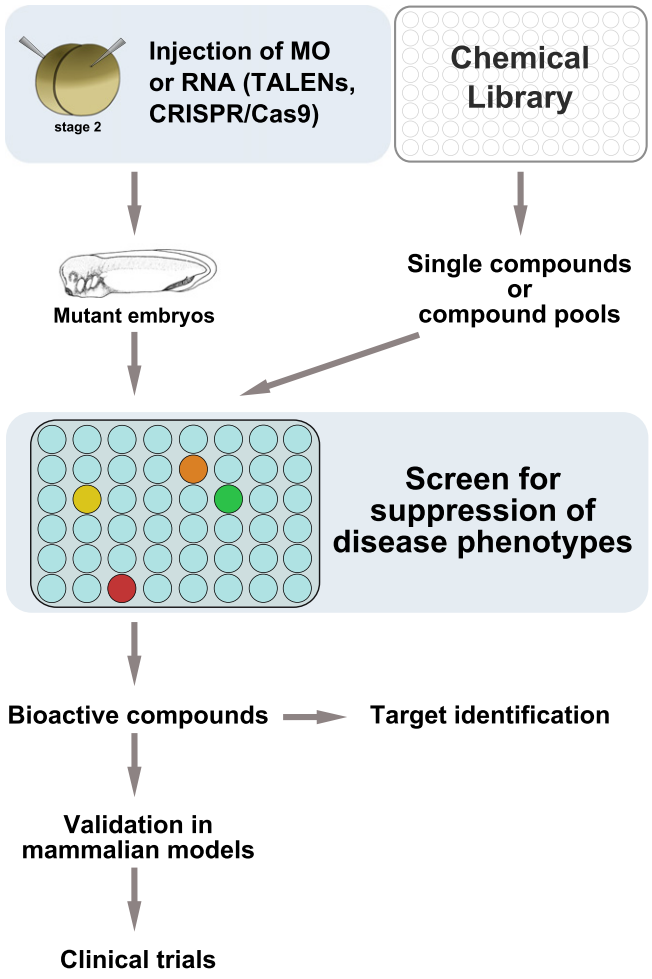

Fig. 7. Engineering *Xenopus* embryos for drug discovery. Embryos at the two-cell stage are injected with morpholino oligonucleotides (MO) or RNA encoding TALENs or CRISPR/Cas components to generate models of inherited human diseases. Mutant embryos by arrayed in multi-well dishes, typically in a 48-well format. Each well receives a chemical compound or a compound pool from a chemical library. Hits are selected for their ability to reverse in vivo the pathological disease phenotype to the wild-type phenotype. Bioactive compounds with unknown targets may require target identification and thus lead to novel targets. Promising compounds will be tested in suitable mammalian disease models, such as transgenic mice. Depending on potency and safety validated and optimized drug candidates will advance to testing in clinical trials in human patients.

Given the rapid improvements made in the precision and targeting specificities and the growing number of reports describing efficient introduction of biallelic mutations using TALENs and CRISPR/Cas technologies in *Xenopus* embryos [109,135], it is conceivable that chemical screens may in future be carried out directly in the F0 generation. There is however undoubtedly much more work needed to evolve precision genome engineering to meet such high demands.

Gene editing methods relying on targeted nucleases have emerged only recently and thus there are no examples in the literature, neither for zebrafish nor for *Xenopus*, demonstrating the massive potential of these tools in phenotypic drug discovery. All the phenotypic drug discovery screens carried out to date in *Xenopus* mentioned in Table 5 have utilized wild-type *X. laevis* embryos. While several *X. tropicalis* mutants recovered from genetic screens have had the lesions mapped to human gene orthologs [65–67], and these mutants have not yet been employed in phenotypic drug screening. The *muzak* mutant carries a missense mutation in the cardiac myosin heavy chain gene *myh6* that creates a premature stop codon resulting in the synthesis of a truncated protein of 1062 amino acids [66]. This mutant is of particular interest as it represents the first *Xenopus* mutant of a rare human inherited disease gene ortholog. Individuals with heterozygous mutations of MYH6 were found to develop either familial hypertrophic cardiomyopathy 14 (OMIM #613251) or dilated cardiomyopathy 1EE (OMIM #613252) [179]. It remains to be determined whether heterozygous *X. tropicalis muzak* mutants will develop late-onset hypertrophic or dilated forms of cardiomyopathy.

Besides chemical-induced *X. tropicalis* mutants, a few examples of transgenic *X. laevis* models of inherited human diseases, primarily retinal degenerative diseases, have been developed in the past [180] and are discussed above (see Section 4.6.) in detail. While the transgenic *Xenopus* models of RP represent invaluable tools to study the disease mechanisms underlying autosomal dominant retinal degenerative diseases, they have to date not been exploited for pharmacological drug screening purposes.

In zebrafish, several chemical screens to identify small molecules that suppress disease phenotypes in mutant lines have been reported [8,181]. For example, the screening of a 5000-compound chemical library for small molecules that rescue the aortic coarctation defect in zebrafish *gridlock* mutants led to the identification of a novel class of organic molecules capable of suppressing the mutant phenotype in vivo [182]. These compounds restored blood flow in the tail by upregulating VEGF expression. The *gridlock* mutation localizes to the transcription factor *hey2* gene [183], which appears to have different functions in mammals. No human patient carrying *HEY2* mutations have been reported to date and in mice, the loss of *Hey2* gene function does not cause aortic coarctation or deficiencies, but instead leads to massive postnatal cardiac hypertrophy with high lethality [184]. Cao and colleagues screened a small custom library of 115 compounds targeting different signaling pathways to identify those that suppressed the body axis curvature defect, which is present in *pkd2* mutant zebrafish [185]. Zebrafish *pkd2* is an ortholog of the human PKD2 gene, which is responsible for one form of autosomal dominant polycystic kidney disease (ADPKD, see 6.3. below for details). The authors identified histone deacetylase (HDAC) inhibitors, including valproic acid (VPA), as suppressors of the zebrafish-specific body axis phenotype. Interestingly, treatment of a mouse model of ADPKD with VPA improved kidney function and reduced cyst formation, eliciting HDAC inhibitors as potential drug candidates for treating ADPKD. In the final example, Peal et al. (2011) devised a chemical screen for compounds that could suppress genetic long QT (LQT) syndrome type 2 (OMIM #613688), a life-threating disorder caused by mutations in the KCNH2 gene [186]. Using the zebrafish *kcnh2* mutant *breakdance*, they discovered 2-methoxy-N-(4-methylphenyl) benzamide (2-MMB) as a compound that reproducibly suppressed the genetic cardiac arrhythmia phenotype. 2-MMB represents a novel reagent for the study of cardiac electrophysiology and could serve as a lead compound for the development of novel drugs to treat human LQT patients. Collectively, these studies underscore the potential of zebrafish mutants for drug discovery. It is important to note that most of the chemically-induced zebrafish mutants employed in these drug screening campaigns do not represent genuine models of

Table 5
In vivo phenotypic chemical screens performed using *Xenopus* embryos.

Phenotypic readout	Protocol type	Chemical libraries	Compounds tested	Positive hits	References
Pigmentation	One-tiered visual screen	Diversity set (NCI) Gen-Plus (Microsource)	1990 960	39	[161]
Edema formation; angiogenesis, lymphangiogenesis	Two-tiered screen (visual followed by in situ hybridization)	LOPAC (Sigma-Aldrich)	1280	32	[159]
Heterotaxia, left–right asymmetry	One-tiered visual screen	Non-commercial compound library	130	1	[158]

rare inherited human diseases or fail to phenocopy accurately the human disease pathologies. With the recent introduction of sophisticated gene editing technologies, the development of precise non-murine models of inherited human diseases becomes now for the first time a viable possibility. These new models are anticipated to reproduce more faithfully clinical phenotypes and thus improve their validity as screening platforms for drug discovery.

6.3. Arguments for developing Xenopus models of rare inherited diseases

Many zebrafish models of human disease have been generated and studied in recent years, ranging from hematological disorders to neurodegenerative diseases, and they provide platforms to accelerate the development of new therapies [181]. Given the emergence of zebrafish as the predominant non-mammalian vertebrate model for whole-organism based drug discovery screening [5,187], one may ask whether there is still a need to develop a second non-mammalian animal model for similar purposes.

As we have outlined above, *Xenopus* frogs are tetrapods and therefore share a longer evolutionary history with humans, which manifests in many more similarities ranging from organ development to conserved physiological functions. Unlike the paleotetraploid genome of zebrafish, the genome of *X. tropicalis* is truly diploid, which simplifies gene targeting approaches as homeologs are not a potential source of genetic redundancy. This is illustrated by a cross-species comparison of the genes responsible for autosomal dominant polycystic kidney disease (ADPKD; OMIM #173900 & #613095), a life-threating human genetic disorder caused by mutations in the polycystin genes PKD1 (OMIM #601313) and PKD2 (OMIM #173910) [188]. Manifestation of ADPKD occurs later in life, which is explained by a two-hit hypothesis of pathogenesis [189] borrowed from oncology (Knudson hypothesis for retinoblastoma). Thus, ADPKD patients carry a congenital mutation (*first hit*) in one of the two PKD1 or PKD2 alleles, respectively. A subsequent somatic mutation (*second hit*) is required later in life to produce renal cyst development. Loss of heterozygosity of PKD genes is therefore the molecular mechanisms underlying cyst formation in ADPKD patients [190]. PKD1 mutations account for the majority of ADPKD cases in human patients. The genomes of *X. tropicalis* and *X. laevis* harbor both a single pkd1 gene each (S.M. Schmitt & A.W. Brändli, unpublished observation), whereas two pkd1 genes are present in zebrafish [191]. The phenotypes of pkd1 mutant *Xenopus* are presently unknown, but in zebrafish the combined knockdown of both pkd1 homeologs was required to reveal the full spectrum of pkd1 loss-of-function phenotypes [191]. Furthermore, mutant disease phenotypes in *Xenopus* models of inherited diseases are more likely to be similar to those seen in mouse models and human patients as partial phenotypes due to sub- and neofunctionalization of homeologs can be ruled out. This is exemplified by a comparison of the phenotypes of zebrafish and *Xenopus* embryos deficient for the PKD2 gene, which accounts for 5% of the patients suffering from ADPKD [192]. Homozygous *Pkd2* mutant mice exhibit laterality defects, severe cardiac malformations, and cystic kidneys, which cause lethality between E13.5 and parturition [193]. Zebrafish lines carrying mutations in the *pkd2* gene have been reported [194,195]. Surprisingly, these *pkd2* mutant embryos do not develop pronephric cysts, but they manifest with defective left-right asymmetry and a very prominent body curvature phenotype resulting from abnormal notochord morphogenesis [191,194]. By contrast, MO-mediated knockdown of the only pkd2 gene present in the *X. laevis* genome resulted in embryos with laterality defects and a highly penetrant pronephric cyst phenotype, whereas no defects in axial morphogenesis were observed (M. Gull, S.M. Schmitt & A.W. Brändli, unpublished observation). The reasons for the differences in the phenotypes seen between mouse and zebrafish Pkd2 mutants are unclear. It is evident that the *Xenopus* pkd2 mutant phenotypes more closely related to those seen in the corresponding mouse Pkd2 mutants.

Finally, the presence of human disease gene orthologs in zebrafish and *Xenopus* needs to be considered. Comparison of the human reference genome has revealed that approximately 70% of human genes have at least one obvious ortholog in the zebrafish genome [39]. This implies that 30% of the human genes are not represented in the zebrafish genome and therefore cannot be studied in this model organism. This also limits the utility of zebrafish to model human inherited diseases. By contrast, the *X. tropicalis* genome was estimated to contain orthologs to approximately 80% of the currently known human disease genes [16]. Based on our own observations, these numbers may in fact be higher. We found in a large-scale comparative study of human genes causing renal tubulopathies that 46 out of 50 genes (92%) studied had orthologs in the *X. laevis* genome (A. W. Brändli, unpublished observation). Furthermore, we found important human disease genes that do not have clearly identifiable zebrafish orthologs but are present in the two *Xenopus* genomes. These include AQP2 (nephrogenic diabetes insipidus, OMIM #125800), BRCA1 (susceptibility to familial breast-ovarian cancer-1, OMIM #113705), FOXC2 (lymphedema-distichiasis syndrome, OMIM #153400), and PKHD1 (autosomal recessive polycystic kidney disease, OMIM #263200). On the basis of the limited examples presented above, it is evident that *Xenopus* represents an indispensible non-mammalian vertebrate model for the study of human disease genes and the development of novel therapeutics.

7. Conclusions

The potential of delivering compounds with therapeutic properties fuels the attractiveness of carrying out drug screens in zebrafish and *Xenopus* taking advantage of their small free-living embryos. Small molecules that can modify or suppress the disease phenotype in embryonic models of rare inherited human disorders will be advanced to preclinical testing in appropriate mouse models and may translate into novel therapies for human patients. In the present review, we have laid out a road map for developing *Xenopus* models of rare inherited human diseases taking advantage of novel customized endonuclease technologies that are rapidly becoming indispensable tools for genome editing in vertebrate model organisms. Recently, TALENs and CRISPR/Cas have emerged as the most powerful technologies due to their simplicity, flexible design principles, rapid implementation possibilities, and impressive gene targeting efficiencies with low off-target effects. These technologies are transforming our abilities to engineer animal models that previously had been refractory to the generation of targeted gene knockouts and the introduction of site-specific single nucleotide changes. In addition, TALENs and CRISPR/Cas technologies will offer novel opportunities for *Xenopus* as a tetrapod animal model facilitating the functional characterization and prioritization of candidate genes identified in human cohort-based genetic studies and genome-wide association studies (GWAS).

When coupled with high-throughput animal models such as zebrafish and *Xenopus*, mutant lines of human inherited diseases can now be precisely engineered for in vivo phenotypic drug discovery screening. Phenotypic drug screening in whole organisms is usually more physiologically relevant and less artificial than in vitro approaches. One disadvantage of drug screening using in vivo models includes the loss of potential lead compounds due to poor drug absorption. Poor relevance of some animal models to human diseases and species-specific differences in drug metabolism are other factors that can contribute to the failures of drug candidates in late stages of the drug development process. The novel genome editing technologies will enable the development of more accurate animal models of human inherited diseases. Furthermore, an increased use of *Xenopus*, which is in evolutionary terms unambiguously less distant to human than zebrafish, will reduce the likeliness of failure of drug candidates once they reach preclinical testing.

Ultimately, the success of non-murine model systems will be measured by their ability to deliver new drug candidates to the clinic. A growing number of chemical screens have in recent years been successfully performed in zebrafish and *Xenopus*. However, there is only one drug candidate, prostaglandin E2 (PGE2) that has not only entered but

still remains in clinical trials. PGE2 was identified by screening a library of well-characterized drugs for compounds that enhance hematopoietic stem cell formation in zebrafish embryos [196]. The efficacy of treating umbilical cord blood with PGE2 to improve engraftment was successfully tested in a Phase I trail and a Phase II trial is under way [197]. The discovery of PGE2, a drug approved to induce labor during childbirth, as novel modulator of HSC homeostasis highlights the power of in vivo phenotypic drug screens to identify new indications for known drugs, a feature that is particularly useful for diseases without effective therapies. The repurposing of approved drugs for new indications can save time and resources in drug discovery and development, while reducing the risk of failure in early clinical trials. This approach is particularly attractive for rare inherited diseases, where effective drugs are urgently needed to treat small patient groups at the lowest cost possible.

In summary, we anticipate that the engineering of *Xenopus* models of inherited human diseases will complement the murine ones in the near future. Hence, *Xenopus* is emerging as another powerful vertebrate model organism enhancing our knowledge of the pathogenic mechanisms of human inherited diseases and providing invaluable platforms for the development of new therapeutics.

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