# 14 Advances in Genome Editing Tools

*Marko E. Horb, Anita Abu-Daya, Marcin Wlizla, Anna Noble, and Matt Guille*

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## 14.1. GENERATION OF TRANSGENIC *XENOPUS*

### 14.1.1. Beginnings

Transgenic animals carry exogenous DNA integrated into their genome and can be designed to fulfill diverse experimental objectives, from using fluorescent proteins to label specific cells/tissues or to observe activity of signaling pathways to using dominant-negative and constitutively active mutants to disrupt gene activity. Prior to the development of stable transgenic lines, experiments to track or disrupt gene function were carried out via microinjection of RNA, DNA, morpholino oligonucleotides, or antibodies and through treatments with small molecules, but these all have related limitations. The mosaic distribution and progressive dilution with every cell division of microinjected reagents typically limits their use to the study of genes involved in early development (Amaya, 2005). Although small molecule treatments can be applied at any stage of development, care must be taken that the molecules used are specific to the genes being disrupted and that the phenotypes observed are not due to a disruption of a broader range of targets than intended (Vogt et al., 2011). These experimental limitations can be circumvented using transgenics. The current transgenic toolset available to *Xenopus* researchers permits efficient generation of stable, non-mosaic animal lines and inclusion of elements for temporal control of transgene activity. These allow their use through all stages of development and make possible the design of transgenic lines able to disrupt gene activity in a highly specific manner.

It has been over three decades since the initial reports that exogenous plasmid DNA was integrated into the *Xenopus* genome following its microinjection into fertilized eggs with successful transmission through the male germline (Etkin and Pearman, 1987; Etkin and Roberts, 1983; Rusconi and Schaffner, 1981). However, microinjection of linearized plasmid DNA is not a practical approach for generation of stable transgenic lines; it is highly inefficient, with only 1% of injected F0 animals showing mosaic integration of the foreign DNA into their germlines (Yergeau et al., 2010). Innovation has followed three distinct ways to improve the transgenic methodologies in *Xenopus*: first, development of alternative approaches to make genomic integration more efficient; second, by streamlining the generation of transgenic plasmids carrying the exogenous DNA; and third, by incorporating elements for temporal regulation of transgene activity, thus expanding on the versatility of this technology. This chapter outlines technological advances in *Xenopus* transgenesis, followed by an overview of how transgenics have impacted research using this popular model organism.

### 14.1.2. Technology Development

Restriction enzyme-mediate integration (REMI) was the first method that made *Xenopus* transgenesis practical (Kroll and Amaya, 1996). In REMI, transgenic plasmid DNA is incubated together with isolated sperm nuclei, a restriction enzyme, and *Xenopus* interphase egg extract and then injected into mature, unfertilized eggs. One to 16% of the injected eggs survive past feeding tadpole stages. However, this is not really a complication, as it is possible to inject

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thousands of eggs at a time, and of those normal survivors, as many as 36% show stable, non-mosaic transgene expression. Furthermore, F0 animals that show strong non-mosaic expression almost invariably transmit the transgene through their germline (Marsh-Armstrong et al., 1999). This was a major improvement in transgenic efficiency over the results obtained by injection of linearized plasmid DNA into fertilized embryos and also works efficiently in *Xenopus tropicalis* (Offield et al., 2000). The major limitation of REMI is that it is highly mutagenic, capable of generating up to four different transgene integration sites with multiple integrations in the injected individual, complicating interpretation of phenotypic analyses (Bronchain et al., 1999; Marsh-Armstrong et al., 1999). Furthermore, studies analyzing late developmental stages are not trivial in the F0 generation due to the small number of survivors (Chesneau et al., 2008). In 2000, REMI was simplified by eliminating the need for restriction enzyme and egg extract while retaining similar efficiency (Sparrow et al., 2000).

Following REMI, the successful innovations in transgenesis techniques focused on increasing rates of survival and normal development, mainly through injection of transgenic reagents into embryos rather than eggs, resulting in less technically demanding procedures and greater normal development. First, a relatively simple method relying on co-injection of the commercially available rare-cutting meganuclease, I-SceI, and transgenic DNA carrying the 18 base-pair long I-SceI recognition site was efficient in both *Xenopus laevis* and *Xenopus tropicalis* (Pan et al., 2006). The observed survival rate past metamorphosis was greater than 55%, with 10% to 12% of the survivors showing non-mosaic transgene expression (Pan et al., 2006). Furthermore, only one to eight copies of the transgene integrated at up to two distinct integration sites.

A second similarly simple technique involves microinjection of mRNA encoding the φC31 bacteriophage integrase with a transgenic plasmid DNA containing a 34 base-pair long bacterial attachment site, *attB* (Allen and Weeks, 2005; Li et al., 2012). The integrase mediates recombination between the *attB* site and a 39 base-pair long phage-dependent attachment site *attP*; recombination, however, can also occur at pseudo-*attP* sites with similarity as low as 24% to the phage *attP* sequence, though at much lower efficiencies (Groth et al., 2004; Thyagarajan et al., 2001). The main advantage of this approach is that it is thought to result in integration of a single transgene into the *Xenopus* genome, although reports of successful germline transmission are lacking (Allen and Weeks, 2005; Li et al., 2012). The European *Xenopus* Resource Centre (EXRC) has an engineered transgenic line that contains an *attP* docking site within a functional cyan fluorescent protein coding sequence. The use of this line in conjunction with φC31 integrase may provide a highly efficient way to generate novel transgenics, screened by loss of cyan fluorescence (Horb et al., 2019).

Finally, two approaches based on the use of transposase-driven transgene integration, using *Sleeping Beauty* or *Tol2*, have been effective in *Xenopus*. Both involve co-injection of the transposase, either as mRNA or protein, with the transposon or DNA encoding the transgenic package flanked by transposase target sequences (Hamlet et al., 2006; Shibano et al., 2007; Sinzelle et al., 2006; Yergeau et al., 2009). Uniquely, this method can also be used in a variant of gene and enhancer trap, in which forward genetic experiments are achieved by secondarily remobilizing the transgene following reintroduction of the transposase (Lane et al., 2013; Yergeau et al., 2011a, 2012).

### 14.1.3. pTransgenesis: Streamlining Transgene Construction

Besides novel ways of promoting transgene integration into the *Xenopus* genome, a major innovation came through use of modular Gateway cloning to streamline the generation of transgenic plasmids. The pTransgenesis system uses multisite Gateway technology to allow for rapid generation of transgenic plasmids via recombination of a destination vector containing DNA sequences required for genome integration and three entry clones, a fluorescent transgenesis reporter, a promoter, and a coding sequence (Love et al., 2011b). The destination vectors allow a choice among transgenic methods, including I-SceI meganuclease, Tol2 transposase, and φC31 integrase. Novel entry clones can be easily generated through simple recombination of a PCR product and donor vector, and existing entry clones can be mixed and matched to generate a diverse range of transgenic plasmids, making this a very powerful and flexible system.

There are, however, a number of issues with this system. First, the particular donor vectors used to generate pTransgenesis entry clones stopped being commercially available shortly after publication. Nonetheless, they can be procured from the Zebrafish International Resource Center (ZIRC) as part of the Tol2kit used for transgenic plasmid creation in zebrafish (Kwan et al., 2007). Second, only three of the four destination vectors contain chicken beta-globin HS4 insulator sequences that have been shown to reduce integration site effects on transgene expression (Allen and Weeks, 2005). Third, one of the vectors includes two I-SceI target sequences flanking the transgene, which may increase the efficiency of transgenesis but risks integration of the vector backbone independent of the transgene sequence. Fourth, the pTransgenesis system, as constructed, is not compatible with the *Xenopus* ORFeome (Grant et al., 2015). The recombination sites used in the ORFeome constructs are the same ones as those used in the pTransgenesis promoter entry plasmids and combining both systems misplaces the ORF within the transgenic construct. Instead, a newer two-plasmid system can be used in conjunction with the ORFeome to rapidly generate transgenic plasmids (Sterner et al., 2019). Like pTransgenesis, this system is versatile and permits selection of the transgenic technique: I-SceI, Tol2, and φC31. One of the destination vectors, pDXTR, allows for rapid gateway recombination with the ORFeome plasmids and includes the Tet-On system for inducible transgene expression. However,

it requires the use of restriction enzyme-based or Gibson Assembly cloning to introduce the promoters of interest (Das and Brown, 2004a; Kerney et al., 2012; Rankin et al., 2011). Alternatively, another destination vector, pDXTP, can be used in conjunction with pDXTR. pDXTP is compatible with the promoter entry vectors included in pTransgenesis, allowing rapid recombination of the promoter upstream of the doxycycline inducible transcription factor rtTA, thus maintaining the ability to use the Tet-On system to control transgene expression. One shortcoming of the pDXTR and pDXTP plasmids is that they are relatively large, and both contain several insulator sequences. This makes pDXTR particularly prone to self-recombination following transformation into *E. coli*, which can be prevented by growing the transformed bacteria at a lower temperature.

The ability to regulate transgene activity in both time and space expands the breadth of experimental questions that can be investigated using transgenics in *Xenopus*. A number of cell- and tissue-specific promoters have been characterized and used for spatial regulation of transgene activity in *Xenopus* (Horb et al., 2019). In addition to the Tet-On system, temporal control of transgene expression in *Xenopus* has been achieved using the temperature-inducible *hsp70* promoter or the modified dual-component GAL4-UAS system with the GAL4 fused to the ligand binding domain of the progesterone receptor (PR) (Beck et al., 2006; Horb et al., 2019). Finally, transgenic lines expressing Cre have been used as drivers to induce switches in fluorescence following a cross to lines with *loxP* sites (Roose et al., 2009; Waldner et al., 2006). These diverse methods of transgenesis, transgenic vector construction, and transgene activity regulation provide a solid framework within which *Xenopus* researchers can design experiments to study biological processes through genome modification.

## 14.2. USES OF TRANSGENIC *XENOPUS*

The way transgenesis would expand the experimental toolkit in *Xenopus* was made evident with the first transgenesis paper by Kroll and Amaya, in which they examined the temporal requirement of FGF signaling in early *Xenopus* development. Previous experiments suggested that FGF signaling was necessary for primary mesoderm induction as well for later processes, including maintenance of mesoderm fate and neural induction and patterning. Mesoderm induction occurs quite early in embryogenesis and can be studied using mRNA injections, but later events require FGF signaling to be perturbed after mesoderm induction. Transgenesis was used to express a dominant negative FGF receptor after mesoderm induction and showed that while FGF was required for maintenance of mesoderm fate, it was not required for neural induction and patterning (Kroll and Amaya, 1996). Its lack of involvement in neural induction contradicted previous data, revealing how transgenics could improve data quality in the frog. In addition to dominant negative protein expression, other loss-of-function approaches, such as shRNA, have also proven effective in *Xenopus* transgenics (Edholm and Robert, 2018).

Using inducible or tissue-specific promoters allows research on developmental processes that occur several days to months after fertilization, including regeneration and metamorphosis. Regeneration is thought to occur through the reactivation of the same program involved in normal development, but this cannot be studied in traditional knockout experiments, since the target tissue/organ may be perturbed by loss of function early in development. In *Xenopus*, tadpole tails regenerate upon amputation from three days after fertilization until metamorphosis, except during a short refractory period at four to five days. Using a heat shock-inducible *hsp70* promoter, Beck et al. (2003) showed that reactivation of the BMP and Notch signaling pathways during this refractory period promoted regeneration, whereas their inhibition at other stages blocked regeneration. In F0 transgenics, they found variability in this ability, possibly due to integration site and transgene expression levels, but in F1 transgenics, the phenotype was more consistent. These results suggested that generating stable transgenic lines produces more robust results.

Metamorphosis in *Xenopus* is a model for human perinatal endocrinology when multiple hormones regulate many aspects of tissue growth, development, remodeling, and maturation (Buchholz, 2015). This late-stage event occurs 30–60 days after fertilization, and transgenesis in *Xenopus* was essential for *in vivo* functional studies (Marsh-Armstrong et al., 2004; Mukhi et al., 2008, 2009; Schreiber et al., 2001). In particular, binary-inducible transgenic systems have proven useful for temporal and tissue-specific control of transgenes during metamorphosis (Buchholz, 2012; Das and Brown, 2004b). The tetracycline (Tet)-inducible system allows for tight control of transgene expression by simple addition of doxycycline (Dox) to the water. This system requires two different transgenes: one promoter (tissue-specific or ubiquitous) to control expression of rtTA (a Dox-dependent transcription factor) and a second tetracycline-inducible (TRE) promoter to control expression of the gene of interest. This system elucidated several aspects of metamorphosis, including gene switching, transdifferentiation of pancreatic acinar cells to ductal cells, and limb development (Brown et al., 2005; Cai et al., 2007; Mukhi and Brown, 2011; Mukhi et al., 2010).

Another benefit of *Xenopus* transgenics is the ability to use promoters from other species, including rat, mouse, and zebrafish, to drive expression in a tissue-specific manner (Beck and Slack, 1999; Love et al., 2011a). This can be used to study the ability of factors to convert one tissue to another. Combining such expression with a secondary reporter to label the tissue generated allows monitoring of transdifferentiation events in real time. For example, using a murine transthyretin promoter to drive expression of pancreatic transcription factors in the liver, combined with the rat elastase promoter driving GFP, it was found that only two pancreatic transcription factors, Ptf1a and Pdx1, were able to convert liver to pancreas (Horb et al., 2003; Jarikji et al.,

2007). First, both *Xenopus* and murine *pdx1* genes acted similarly in converting liver to pancreas in the *Xenopus* tadpole, but both proteins required an extra VP16 activation domain. The ability of other pancreatic transcription factors was tested similarly, and only one other transcription factor, Ptf1a, had similar activity. These results reveal the power of using multiple transgenes to test the functional ability of different factors *in vivo* in a tissue-specific manner allowing for analysis of cell fate conversions and commitment.

One of the major embryological benefits of *Xenopus* is transplantation that creates chimeric embryos, and transgenic *Xenopus* embryos have proven very useful for these experiments. Creating chimeric embryos between wild type and transgenic embryos has been used to study pancreas development, regeneration, and the origin of muscle satellite cells (Daughters et al., 2011; Gargioli and Slack, 2004; Jarikji et al., 2009). The developing pancreas is derived from separate dorsal and ventral buds arising from the roof and floor of the archenteron. These buds can be selectively labeled in chimeric embryos of wild type and pElas:GFP transgenics. Using such an approach, Jarikji et al. (2009) found that the ventral pancreatic cells migrate into the dorsal pancreas after fusion, whereas the dorsal pancreatic cells do not. In another example, grafting specific regions from a transgenic CMV:GFP neurula stage embryo onto a wild type host enabled Gargioli and Slack (2004) to follow the fate of individual tissues (neural, notochord, or somites) during tail regeneration. They found that notochord and spinal cord regenerate from the same tissue, whereas muscle cells regenerate from a small population of satellite cells. Other lines that are beneficial for such experiments include ROSA26:GFP and Brainbow lines for long-term fate mapping (Gross et al., 2006).

In addition to following transplanted cells, transgenes can be used to isolate specific embryonic cells or nuclei, facilitating analysis of transcriptomes or proteomes. One method uses two transgenes, one labeling nuclei of target cells with a biotin ligase receptor and the second expressing the BirA biotin ligase to biotinylate the target nuclei for isolation. Using cell-type-specific DNA elements, this approach generated proteomic profiles of *Xenopus* cardiac nuclei (Amin et al., 2014). Transgenic *Xenopus* in which particular cell types are labeled were used to purify these cells for downstream analysis; this has relied on the speed and simplicity of disaggregating cells in *Xenopus* prior to FACS sorting, which was combined with RNAseq to identify key regulators of tail regeneration (Kakebeen et al., 2020).

Although these transgenic lines allow the biochemistry of development to be studied, there are others that focus more on cell biology. Some lines label various subcellular structures, while others allow real-time spatiotemporal analysis of signaling pathway outputs, including Wnt, calcium, epigenetic changes, and oxidative stress (Love et al., 2013; Offner et al., 2020; Suzuki et al., 2016; Takagi et al., 2013; Tran and Vleminckx, 2014).

Successful transgenic strategies depend on having identified the appropriate DNA control elements to drive expression of transgenes in the required temporal and spatial manner. This has proven the most challenging element of transgene design despite all of the information now available regarding epigenetic modification and conservation of genomes (Kim et al., 2019). While BAC and fosmid transgenesis have been used successfully to address this challenge (Fish et al., 2011; Ochi et al., 2012), gene editing, which is discussed in the following, has produced an alternative approach that avoids the challenges of handling large, fragile constructs and the possibility of extremely distant control elements: inserting a transgene into the endogenous locus so that its expression is controlled by all of the DNA elements and epigenetic mechanisms that regulate the gene normally.

## 14.3. GENETICS AND GENE EDITING IN *XENOPUS*

Traditionally, *Xenopus* were used mainly for embryological experiments, with limited use as a genetic model. This was largely due to the allotetraploidy of *X. laevis*, which was the species used until recently; its long time to sexual maturity; and the resulting amount of effort required to breed successive generations. The publication of the *X. tropicalis* genome in 2009 and the *X. laevis* genome in 2016 together with new genome editing technologies that have become available in the last seven years have led to the generation of many new *Xenopus* mutants. In this section, we outline a brief history of *Xenopus* mutants and give an overview of gene editing successes in *Xenopus* and future directions.

### 14.3.1. Beginnings

Prior to the modern era of gene editing, naturally occurring *Xenopus* mutants were identified in the laboratory through successive inbreeding or gynogenetic screens. Several naturally occurring mutations were identified and studied, including *anucleate*, which lacked nucleoli and was instrumental in cloning ribosomal RNA genes (Elsdale et al., 1958; Wallace, 1960). The second spontaneous *X. laevis* mutant to be identified was the *periodic albinism* mutant, which produces white/yellow embryos that are excellent for gene expression analysis and commonly used by the *Xenopus* community (Hoperskaya, 1975). Recently, this mutation was mapped to a 1.9kb deletion in the *hps4* (Hermansky Pudlak syndrome type 4) gene (Fukuzawa, 2021). Other developmental mutants were identified in offspring derived from nuclear transfer animals at the Geneva *Xenopus* Centre (Droin, 1992). These naturally occurring mutants set the foundation for future genetic studies in *Xenopus*.

### 14.3.2. *X. tropicalis* and Forward Genetic Screens

Interest in *Xenopus* genetics was reignited after the introduction to the laboratory of a closely related species, *Xenopus tropicalis* (Abu-Daya et al., 2012). *X. tropicalis* is the only diploid species in the genus, with one of the smallest known haploid genomes, $1.5 \times 10^9$ bp in 10 chromosomes ($2n = 20$).

Genetic screens in zebrafish have yielded many insights into vertebrate developmental biology, but the realization that the teleost genome had undergone a duplication event generated interest in developing a genetic model organism with a more canonically organized genome that was evolutionarily closer to mammals (Glasauer and Neuhauss, 2014). In 1999 at the NIH Non-Mammalian Model Meeting, *Xenopus* was one of the model organisms discussed, and subsequently consensus was reached on ten priority areas for large-scale genomic and genetic resource development for *Xenopus*. These included establishing the viability of *X. tropicalis* as a genetic model organism through pilot genetic screens, including chemical mutagenesis, large-scale radiation-induced deletions, and insertional mutagenesis, and developing resources such as a genetic map and sequencing the *X. tropicalis* genome (Klein et al., 2002). As a result, a number of laboratories undertook forward screens for mutations affecting *X. tropicalis* development (Goda et al., 2006; Noramly et al., 2005).

Since amphibians do not have imprinting and fertilization is external, it is possible to use gynogenesis to obtain diploid embryos without paternal genetic contributions (Tompkins, 1978). Gynogenesis allows F1 female progeny of F0 mutagenized animals to be screened for recessive mutations without the need to obtain adult F2 animals for sib crosses. The technique is simple: irradiated, macerated wild type testes are used for *in vitro* fertilization; the sperm initiate egg cleavage, but their genetic material is destroyed, creating haploid embryos. Diploidy is restored by cold shock or pressure early after fertilization, which inhibits polar body formation and prevents the loss of maternal chromosomes duplicated during meiosis II (Geach et al., 2012). Disadvantages of gynogenesis are that the frequency of mutation depends on the distance from centromeres, ranging from 50% near centromeres to around 10% near telomeres, not the classical Mendelian ratio of 25%. Also, background abnormal gastrulation is higher than in normal fertilization. Gynogenetic screens thus focus on later development, uncovering mutations that affected organogenesis rather than early patterning. In the pilot gynogenetic screens, over 100 potential mutant phenotypes were observed, including defects in heartbeat, motility, pigmentation, otolith formation, haematopoiesis, gut coiling, axis formation, and left-right asymmetry (Goda et al., 2006; Noramly et al., 2005). Once a candidate mutation was identified in a female, an F2 generation was raised to adulthood to confirm the heritability of the phenotype in classic sib crosses.

The first mutation cloned affected cardiac function (Abu-Daya et al., 2009). Homozygous *muzak* tadpoles had no heartbeat, caused by a nonsense mutation in *myh6*. The resulting premature stop codon caused nonsense mediated decay of *myh6* mRNA, and the lack of myosin heavy chain prevented sarcomere formation in *muzak* cardiomyocytes. Another mutation that affected sarcomere assembly was *dicky ticker;* homozygous embryos were completely paralyzed and had no heartbeat. The genetic lesion was a missense mutation in the muscle-specific chaperone *unc45b*, required for the correct folding of the head domain of heavy chain myosins (Geach and Zimmerman, 2010). A model for human disease was *no privacy*, a recessive, non-lethal pigmentation mutant. The phenotype is characterized by significantly reduced pigmentation; the genetic mutation was identified as a 10 base pair deletion in the *hps6* homologue of the *Hermansky-Pudlak Syndrome 6* gene (Nakayama et al., 2017).

Improved genomic resources sped up positional cloning of mutations. In the five years after the identification of *muzak*, six more chemically induced mutants were mapped. These included *kaleidoscope*, characterized by variegated retinal epithelium and head cartilage defects caused by a splicing mutation in the ATPase copper transporting alpha (*atp7a*) gene, which is implicated in Menkes disease; *white heart*, characterized by haematopoiesis defects caused by a mutation in the *smad4.1* gene; *cyd vicious* characterized by a severe eye phenotype and very poor melanocyte migration from the neural tube, mapped to the *DSIF elongation factor subunit (supt5h)* gene; the otolith formation mutants *komimi*, a splicing mutation in the *otoconin90 (oc90)* gene; and *seasick*, a nonsense mutation in the vesicle transport adaptor protein *ap3d1* (Abu-Daya et al., 2012).

### 14.3.3. TILLING

The screens described previously were all "forward genetic" screens; at the same time, a "reverse genetics" project searched for mutations in specific genes by TILLING (targeting induced local lesions in genomes) using males produced by ENU mutagenesis (Stemple, 2004). Capillary sequencing was initially used to search for mutations in specific genes requested by the *Xenopus* community (Goda et al., 2006). This approach did not produce many mutants, since the F1 males tested were mosaic for mutations due to treating mature sperm, not spermatogonia, with ENU and PCR amplification introduced allele bias. However, a TILLING screen on F1 animals produced by spermatogonial ENU mutagenesis produced a nonsense mutation in the retinal anterior homeobox (*rax*) gene, which resulted in eyeless tadpoles (Fish et al., 2014). With the increasing availability of next-generation sequencing, it became possible to sequence the whole exome of mutants. This new approach uncovered mutations in more than 300 genes, although these were not necessarily in specific genes requested by *Xenopus* researchers.

### 14.3.4. Insertional Mutagenesis

Another strategy to produce *Xenopus* mutants was insertional mutagenesis. If a transgene integrates into a coding sequence or an important regulatory region, it will disrupt the function of that gene. Insertional mutagenesis is attractive because cloning the site of integration is simpler and faster than positional cloning. The basic approach using Sleeping Beauty is described previously, and a pilot study showed that in these "hopper frogs," the transposon was indeed excised and reintegrated. In approximately 80% of

cases, the re-integration was within 3MB of the donor locus (Yergeau et al., 2011b).

Despite these promising initial results, no insertional mutations were produced in these screens. However, a functionally disruptive mutation was discovered accidently when an attempt was made to breed a *Xenopus tropicalis* line, carrying a nkx2.5-GFP insert, to homozygosity (Abu-Daya et al., 2011). Approximately 25% of metamorphic froglets completely lacked forelimbs, including the scapula and clavicle. *In situ* hybridization showed that mutant tadpoles did not express *tbx5* in the prospective forelimb region, although cardiac expression was unaffected. The integration site was cloned by ligation-mediated PCR in the first intron of the *nephronectin* gene, a secreted ligand of Alpha8Beta1, which was necessary for metanephros formation in mouse but had not been implicated in limb generation prior to this point. Early experiments thus showed the potential of using the frog model for genetics experiments; however, before this potential could be transformed into a major screening program as in other models, the landscape was changed by the availability and low cost of large-scale sequencing together with gene editing methods.

### 14.3.5. Reverse Genetics Using dsBreaks

Reverse genetics really became feasible in *Xenopus* with the introduction of new gene editing techniques that allowed targeted induction of double strand (ds) breaks in the genome (Lei et al., 2013; Tandon et al., 2017). The first study used Zinc Finger Nucleases to demonstrate the possibility of targeted mutations in the *Xenopus* genome (Young et al., 2011). The first published studies with TALENS were in 2012, and there have now been many different TALEN mutants created in both *Xenopus* species (2 in *X. laevis* and 14 in *X. tropicalis*) (Ishibashi et al., 2012; Lei et al., 2012). Initially, most of these studies analyzed F0 mutants with mosaic mutations, but phenotypes were successfully observed. The first germline TALEN mutants generated were F1 compound heterozygotes; these *X. tropicalis pax6* mutants displayed phenotypes similar to human aniridia patients, showing how *Xenopus* can be used to model human disease (Nakayama et al., 2015). The first *X. laevis* germline mutant was made using oocyte host transfer to improve mutation rates in both *tyr.L* and *tyr.S* genes (Ratzan et al., 2017). This paper showed that one can generate mutants effectively in the allotetraploid *X. laevis*. The first homozygous null TALEN mutants were generated in the *X. tropicalis* protein arginine histone methyltransferase 1 gene (*prmt1*) (Shibata et al., 2019). Prmt1 knockout mice die shortly after implantation, preventing functional analysis. Since *Xenopus* embryos develop externally, *prmt1*$^{-/-}$ embryos survive, allowing for functional analysis, but show delayed growth after five days and eventually die nine days later. Multiple genes can also be mutated using TALENs. Knockout of *rnf43* and *znf3* together (but not individually) led to limb deformities in F0 *X. tropicalis* (Szenker-Ravi et al., 2018). TALEN use peaked in terms of *Xenopus* publications in 2016, but already it had been overtaken by a different method of making ds breaks.

Shortly after TALENs, the CRISPR-Cas technology became available. The ease with which F0 *Xenopus* crispants can be made, the high level of indels caused, and the strong penetrance of resulting phenotypes led this method to become widely used in *Xenopus*. The first *Xenopus* CRISPR mutants were published in 2013, and in the last five years, over 50 different mutants have been published (Blitz et al., 2013; Nakayama et al., 2013). Due to its diploidy and growth at higher temperatures (where Cas is more effective), most CRISPR mutants have been F0 mosaic mutants generated in *X. tropicalis*; they affect a wide range of processes, including immunology, cancer, kidney, neurogenesis, limb, metamorphosis, regeneration, and eye development (Table 14.1). Examples of using *Xenopus* and CRISPR-Cas to underpin the understanding of human genetic disease include a series of studies analyzing the effects of gene variants causing congenital heart defects by the Khoka lab (Bhattacharya et al., 2015; Deniz et al., 2018). Reversade and co-workers investigated tetra-amelia syndrome, which causes lung aplasia and a lack of limbs. The deletion of *rspo2* by CRISPR-Cas caused amelia, validating the link between the gene and disease. Moreover, deletion of two transmembrane ligases associated with the disease caused formation of ectopic limbs, also revealing a master regulator of limb number (Szenker-Ravi et al., 2018). This example demonstrates how both clinical and discovery research is enhanced by analysis of patient-directed gene knockouts in *Xenopus*. For the RhoGEF TRIO, *Xenopus* crispants targeting one of its two human mutation hotspots was used to link that hotspot with

**TABLE 14.1**
**Genetically Altered *Xenopus* Made Using Targeted Nucleases**

| Gene(s) | Year | Species | F0/F1 | Reference/Notes |
|---|---|---|---|---|
| **ZFNs** | | | | |
| *egfp, nog* | 2011 | tropicalis | F0 | (Young et al., 2011) |
| *tyr* | 2012 | tropicalis | F0/F1 | (Nakajima et al., 2012) |
| *nog* | 2017 | tropicalis | F1 | (Young et al., 2017) homozygous null |
| **TALENs** | | | | |
| *tyr* | 2012 | tropicalis | F0 | (Ishibashi et al., 2012) |
| *nog, ptf1a, ets1* | 2012 | tropicalis | F0 | (Lei et al., 2012) |
| *egfp* | 2013 | laevis | F0 | (Sakuma et al., 2013) |
| *tyr, pax6* | 2013 | laevis | F0 | (Suzuki et al., 2013) |
| *ndrg1a* | 2013 | tropicalis | F0 | (Zhang et al., 2013) |
| *tyr, nog, mmp-9.2* | 2013 | tropicalis | F0 | (Nakajima et al., 2013) |
| *tyr, egfp* | 2014 | laevis | F0 | (Sakane et al., 2014) |
| *sp8* | 2014 | tropicalis | F0 | (Chung et al., 2014) agrees with MO data |
| *tyr* | 2015 | tropicalis | F1 | (Nakajima and Yaoita, 2015) deadsouth/germ cell; F0 crossed with ZFN albino |
| *thra* | 2015 | tropicalis | F0/F1 | (Choi et al., 2015) |

(*Continued*)

**TABLE 14.1 (Continued)**

| Gene(s) | Year | Species | F0/F1 | Reference/Notes |
|---|---|---|---|---|
| *tyr, pax6* | 2015 | laevis | F0 | (Miyamoto et al., 2015) oocyte host transfer |
| *cygb* | 2015 | laevis | F0 | (Nakade et al., 2015) |
| *pax6* | 2015 | tropicalis | F1 | (Nakayama et al., 2015) compound het |
| *apc* | 2015 | tropicalis | F0 | (Nieuwenhuysen et al., 2015) |
| *thra* | 2015 | tropicalis | F0 | (Wen and Shi, 2015) |
| *dot1l* | 2015 | tropicalis | F0 | (Wen et al., 2015) |
| *ouro1, ouro2, foxn1* | 2016 | tropicalis | F0 | (Nakai et al., 2016) |
| *thra* | 2017 | tropicalis | F2 | (Choi et al., 2017) |
| *hps6* | 2017 | tropicalis | F1 | (Nakayama et al., 2017) agrees with MO data, ENU or natural mutation |
| *p2ry4* | 2017 | laevis | F0 | (Harata et al., 2019) |
| *mad1* | 2017 | tropicalis | F2 | (Okada et al., 2017) |
| *tyr* | 2017 | laevis | F1 | (Ratzan et al., 2017) L and S mutants from oocyte host transfer. Compound hets |
| *thra* | 2017 | tropicalis | F2 | (Wen et al., 2017) |
| *tbxt/tbxt.2* | 2018 | tropicalis | F1/F4 | (Gentsch et al., 2018) F1 mutants for tbxt; tbxt/tbxt2 double knockouts in F4 |
| *thra/thrb* | 2018 | tropicalis | F2/F1 | (Nakajima et al., 2018) |
| *mecom* | 2018 | tropicalis | F2 | (Okada and Shi, 2018) |
| *pomc* | 2020 | tropicalis | F1 | (Shewade et al., 2020) compound het |
| *prmt1* | 2020 | tropicalis | F2 | (Shibata et al., 2019) |
| *thra* | 2021 | tropicalis | F2 | (Tanizaki et al., 2021b) |
| **CRISPR** | | | | |
| *tyr* | 2013 | tropicalis | F0/F1 | (Blitz et al., 2013) |
| *tyr* | 2013 | tropicalis | F0/F1 | (Nakayama et al., 2013) |
| *cela1.2, ets1, ets2, hspa5, hhex, pgat, pdx1, ptf1a, tm4sf4, tyr* | 2014 | tropicalis | F0/F1 | (Guo et al., 2014) |
| *foxj1, pax8, dnah9, galnt1, ctnnb1* | 2015 | tropicalis | F0 | (Bhattacharya et al., 2015) agrees with MO data |
| *tubb2b, tyr* | 2015 | tropicalis | F0/F1 | (Shi et al., 2015) knockin GFP, ela-GFP |
| *ptf1a, tyr* | 2015 | laevis | F0 | (Wang et al., 2015) agrees with MO data |
| *gsc, tyr* | 2016 | tropicalis | F0/F1 | (Blitz et al., 2016) leapfrogging, F0 and F1 phenotypes |
| *cfap299* | 2016 | laevis | F0 | (Jaffe et al., 2016) |
| *pax3, snai1, ctnnb1, sox9, tfap2a, pax3, zic1, snai2,* | 2016 | tropicalis | F0 | (Liu et al., 2016) |
| *rb1, rbl1* | 2016 | tropicalis | F0 | (Naert et al., 2016) dual knockout |
| *ctnnb1* | 2017 | both | F0/F1 | (Aslan et al., 2017) knock in by oocyte host transfer |
| *xnc genes* | 2017 | laevis | F0 | (Banach et al., 2017) |
| *rho* | 2017 | laevis | F0/F1 | (Feehan et al., 2017) |

(*Continued*)

**TABLE 14.1 (Continued)**

| Gene(s) | Year | Species | F0/F1 | Reference/Notes |
|---|---|---|---|---|
| *tyr* | 2017 | tropicalis | F0 | (Park et al., 2017) base editing |
| *npffr1.1* | 2017 | laevis | F0 | (Waqas et al., 2017) |
| *lhx1, slc45a2* | 2018 | laevis | F0 | (DeLay et al., 2018) |
| *thrb* | 2018 | tropicalis | F0 | (Sakane et al., 2018) |
| *fgfr4* | 2018 | tropicalis | F0 | (Sempou et al., 2018) |
| *tbx5* | 2018 | tropicalis | F0 | (Steimle et al., 2018) |
| *rnf43, znrf3, rspo2* | 2018 | tropicalis | F0 | (Szenker-Ravi et al., 2018) supernumery limbs, linked to clinic |
| *katnal2* | 2018 | tropicalis | F0 | (Willsey et al., 2018) agrees with MO data |
| *xnc10* | 2019 | laevis | | (Banach et al., 2019) cell line knockout |
| *dsp* | 2019 | laevis | F0 | (Bharathan and Dickinson, 2019) agrees with MO data |
| *l1cam, crb2* | 2019 | tropicalis | F0 | (Date et al., 2019) |
| *dnmbp* | 2019 | laevis | F0 | (DeLay et al., 2019) MO as well |
| *tbx4* | 2019 | tropicalis | F0 | (Kariminejad et al., 2019) link to clinic, limb defect |
| *neurod2* | 2019 | tropicalis | F0 | (Sega et al., 2019) link to clinic; RNA overexpression |
| *six1* | 2019 | tropicalis | F0 | (Sullivan et al., 2019) |
| *rpe65, gnat1* | 2019 | laevis | F0 | (Wen et al., 2019) |
| *ctnnd1* | 2020 | tropicalis | F0 | (Alharatani et al., 2020) |
| *trio* | 2020 | tropicalis | F0 | (Barbosa et al., 2020) link to clinic, domain specific effect |
| *pax9* | 2020 | tropicalis | F0 | (Farley-Barnes et al., 2020) |
| *tnnc1* | 2020 | tropicalis | F0 | (Landim-Vieira et al., 2020) |
| *dlg5* | 2020 | tropicalis | F0 | (Marquez et al., 2021) link to clinic |
| *rb1, rbl1, tp53* | 2020 | tropicalis | F0 | (Naert et al., 2020a) cancer |
| *multiple* | 2020 | both | | (Naert et al., 2020b) designing efficient guides |
| *junB* | 2020 | tropicalis | F0/F1 | (Nakamura et al., 2020) compound hets |
| *cfap43, foxj1* | 2020 | laevis | F0 | (Rachev et al., 2020) agrees with MO data |
| *daam2* | 2020 | tropicalis | F0 | (Schneider et al., 2020) link to clinic |
| *thrb* | 2020 | tropicalis | F2 | (Shibata et al., 2020a) |
| *thra/thrb* | 2020 | tropicalis | F2 | (Shibata et al., 2020b) double knockout |
| *adprhl1* | 2020 | laevis | F0 | (Smith et al., 2020) agrees with MO data |
| *ednra, ednrb2, edn1, edn3* | 2020 | laevis | F0 | (Square et al., 2020) |
| *nr3c1* | 2020 | tropicalis | F2 | (Sterner et al., 2020) |
| *ncoa3* | 2020 | tropicalis | F1 | (Tanizaki et al., 2021a) compound hets |
| *gja8, dnase2b* | 2020 | laevis | F0 | (Viet et al., 2020) |
| *Msmb.3* | 2020 | tropicalis | F0 | (Wang et al., 2020) agrees with MO data |
| *mtnr1a* | 2020 | tropicalis | F0/F1 | (Wiechmann et al., 2020) |

(*Continued*)

**TABLE 14.1 (Continued)**

| Gene(s) | Year | Species | F0/F1 | Reference/Notes |
|---|---|---|---|---|
| *dyrk1a* | 2020 | tropicalis | F0 | (Willsey et al., 2020) agrees with MO data |
| *chd1* | 2020 | laevis | F0 | (Wyatt et al., 2020) |
| *cep70* | 2021 | laevis | F0 | (Kim et al., 2021) agrees with MO data |
| **HDR** | | | | |
| *krt12.2, npm3* | 2014 | laevis | F0 | (Nakade et al., 2014) CRISPR/TALEN, TAL-PITCh and CRIS-PITCh |
| *cfap299* | 2016 | laevis | F0 | (Jaffe et al., 2016) plasmid |
| *ctnnb1* | 2017 | both | F0 | (Aslan et al., 2017) CRISPR, oocyte host transfer, oligo |
| *myh6, gapdh* | 2018 | tropicalis | F0 | (Mao et al., 2018) CRISPR, donor plasmid |
| *xnc10* | 2019 | laevis | F0 | (Banach et al., 2019) CRISPR, donor plasmid |
| *slc45a2* | 2020 | both | F0 | (Nakayama et al., 2020) CRISPR, ssDNA |
| **Base editing** | | | | |
| *tyr, tp53* | 2017 | laevis | F0 | (Park et al., 2017) Cas9-linked cytidine deaminase BE3 |
| *tyr, tbx5, apc, cyp1b1, kcnj2, tbx22, gdf5, hhex, sftpb, ptf1a* | 2019 | tropicalis | F0 | (Shi et al., 2019) Cas9-linked cytidine deaminase BE3 |

Legend: Examples of published, genetically altered *Xenopus* show how the number of such animals is growing. Uncommon or novel aspects of particular studies are highlighted in the "Notes" column.

a specific phenotype in TRIO patients, showing that such experiments can extend beyond simple loss of function (Barbosa et al., 2020). Other examples include *tbx4*, *rho*, *neurod2*, *daam2*, and *dlg5* (Feehan et al., 2017; Kariminejad et al., 2019; Marquez et al., 2021; Schneider et al., 2020; Sega et al., 2019).

The first germline CRISPR mutants were compound heterozygotes produced using the leapfrogging technique, which bypasses embryonic lethality by transplanting the endoderm (the germ cells' location) from an F0 mutant into wild type; crossing two F0 *gsc* mutants produced this way resulted in the expected phenotypic mutants (Blitz et al., 2016). Compound mutations in *mtrn1a* in F1 *X. tropicalis* caused rod photoreceptor degeneration, while *junB* F1 mutants showed defective tail regeneration (Nakamura et al., 2020; Wiechmann et al., 2020). The first F2 homozygous null CRISPR mutants were made in genes affecting metamorphosis, including *thra*, *thrb*, and *nr3c1*, and more such homozygous null mutants are in the pipeline (Shibata et al., 2020b, 2020a; Sterner et al., 2020). The biggest drawbacks to making germline mutants in *Xenopus* are the effort, time, and cost needed to raise them through metamorphosis. To increase the generation of germline mutants, the National *Xenopus* Resource (NXR) has embarked on a project to generate over 200 mutants. To date, they have produced over 120 mutants, and they also host visiting researchers to come and work on these mutants or create new mutants. These mutants are cataloged on Xenbase and available to all researchers interested in working with them.

## 14.4. FUTURE DIRECTIONS

CRISPR-Cas gene editing revolutionized site-specific mutation in *Xenopus*, but the use of this new technology to generate site-specific integration is more challenging. Random integration of exogenous DNA through transgenesis works efficiently in *Xenopus*, but site-specific integration of large insertions initially proved difficult to achieve. Initial reports showed such an approach is feasible in *Xenopus*, but the methods were inefficient and unreliable, producing imprecise, mosaic mutations (Aslan et al., 2017; Jaffe et al., 2016; Mao et al., 2018; Nakade et al., 2014; Nakayama et al., 2020). Three of these reports utilized plasmid DNA containing either one or two target sites for sgRNA, allowing cleavage of circular vector in the embryo. In these studies, insertions occurred in both orientations, revealing imprecise integration. In the fourth study, a ssDNA oligonucleotide was injected with Cas9 into oocytes, which were then matured and implanted into another female, and the laid eggs were then fertilized *in vitro*; this is known as the oocyte host transfer (OHT) approach (Aslan et al., 2017). This approach was the most successful, with around 10% precision integration, but only a short ssDNA donor was tested, preventing insertion of fluorescent tags. The problem is that OHT is difficult, and few embryos survive to adulthood. A recent study showed that homology directed repair (HDR) is possible in embryo injections when using long single-stranded DNA (lssDNA) (Nakayama et al., 2020). Though few adult animals were tested for germline transmission, they showed successful insertion of point mutations and fluorescent proteins. One study showed that it is possible, in *X. laevis*, to selectively knock-in constructs into either the L or S homeologue of a gene (Jaffe et al., 2016). The future of HDR insertion in *Xenopus* is to generate novel, tagged proteins to allow for real-time visualization as well to generate precise point mutations to model human disease.

If lssDNA co-injection with CRISPR-Cas9 proves as successful at other loci as in this first report, it may come to dominate making specific, targeted changes to the genome. There are, however, alternatives that avoid making ds breaks but can make small alterations. Base editing involves targeting a Cas9 nickase fused to either a cytidine or adenine deaminase to a specific site in the genome, there converting T-A to C-G or G-C to A-T, respectively. This method has been used successfully in *Xenopus*, producing editing rates of up to 20.5%, but not extensively taken up by the community, most likely due to the limited changes available and the lack of single base-pair accuracy (Park et al., 2017).

Prime-editing is a related method that allows slightly larger changes to be made using Cas9 nickase fused to reverse transcriptase and a complex multifunctional prime-editing sgRNA (Anzalone et al., 2020). At the time of writing, this technique is to our knowledge yet to be used in *Xenopus* with success.

## 14.5. CONCLUSIONS

The range of methods currently available to *Xenopus* researchers for efficient genome modification is extensive and contains robust techniques for both transgenesis and mutagenesis. When combined and used together with other experimental manipulations commonly used in *Xenopus*, such as tissue-targeted microinjection, for example, they permit the pursuit of experimental questions not easily explored in other model systems. Mutations in human *PKD1* are associated with autosomal-dominant polycystic kidney disease (ADPKD), and homozygous mutations or deletions of *pkd1* in mice are embryonic lethal (Blackburn and Miller, 2019). Similarly, CRISPR-Cas9 induced mutations in *X. laevis pkd1* result in F0 edema and eventual lethality (Figure 14.1A). To bypass the embryonic lethality and more specifically target the germline for generation of mutant lines, *pkd1* sgRNAs can be microinjected into the vegetal pole at the 16-cell stage (Figure 14.1B). The effects of *pkd1* mutations on early kidney morphogenesis can, however, be easily observed in *Xenopus* if induced in the *pax8:GFP* transgenic background in which the pronephros is labeled with GFP. This is even more powerful if combined with unilateral mutagenesis where the *pkd1* sgRNA injected side shows disrupted pronephric morphology, and the other side injected with a control sgRNA provides a morphologically normal internal control (Figure 14.1C,D). *Xenopus*, which has always been a powerful system in many respects, has bravely leapt into the heart of the genetic age.

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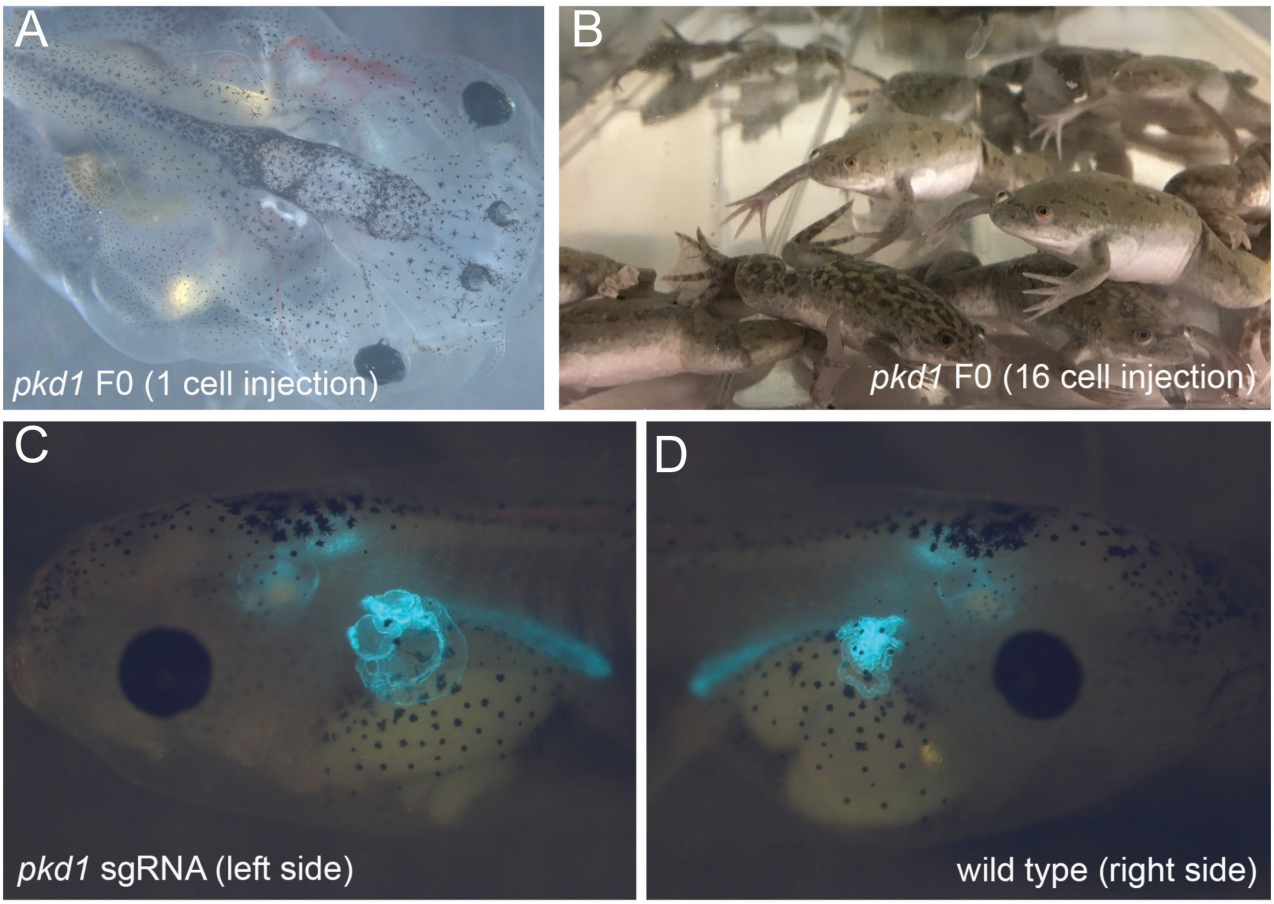


**FIGURE 14.1** CRISPR knockout of *pkd1* in *X. laevis*. (A) Ten-day-old F0 *pkd1* knockout tadpole with severe edema; these tadpoles die before 14 days. (B) Six-month-old F0 *pkd1* frogs generated by injecting the sgRNA into vegetal blastomeres at the 16-cell stage. These F0 tadpoles do not generate edema and survive to adulthood. (C) Left side of *Xla.Tg(pax8:GFP)*$^{Ogino}$ transgenic tadpole injected with *pkd1* sgRNA. Pronephric tubules are dilated. (D) Uninjected right side of tadpole showing normal pronephric tubules.

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